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PATENT APPLICATION

for

CHIMERIC ADENOVIRUSES

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CHIMERIC ADENOVIRUSES

Technical Field: The invention relates to the field of molecular genetics and medicine. In particular the present invention relates to the field of gene therapy, more in particular to gene therapy using viruses, especially adenoviruses.

Background: In gene therapy, genetic information is delivered to a host cell in order to either correct (supplement) a genetic deficiency in the cell, or to inhibit an unwanted function in the cell, or to eliminate the host cell. Of course, the genetic information can also be intended to provide the host cell with a wanted function, for instance to supply a secreted protein to treat other cells of the host, etc.

Thus, there are basically three different approaches in gene therapy, one directed towards compensating a deficiency present in a (mammalian) host; the second directed towards the removal or elimination of unwanted substances (organisms or cells) and the third towards providing a cell with a wanted function.

For the purpose of gene therapy, adenoviruses have been proposed as suitable vehicles to deliver genes to the host. Gene-transfer vectors derived from adenoviruses (so-called adenoviral vectors) have a number of features that make them particularly useful for gene transfer. 1) the biology of the adenoviruses is characterized in detail, 2) the adenovirus is not associated with severe human pathology, 3) the virus is extremely efficient in introducing its DNA into the host cell, 4) the virus can infect a wide variety of cells and has a broad host-range, 5) the virus can be produced at high virus titers in large quantities, and 6) the virus can be rendered replication defective by deletion of the early-region 1 (E1) of the viral genome (Brody et al, 1994). However, there are

still drawbacks associated with the use of adenoviral vectors. Typically, adenoviruses, especially the well investigated serotypes usually elicit an immune response by a host into which they are introduced. Also, although the virus generally ~~spoken~~ SPEAKING has a wide infection range, there is a problem in targeting certain cells and tissues. Also, the replication and other functions of the adenovirus are not always very well suited for the cells which are to be provided with the additional genetic material.

The adenovirus genome is a linear double-stranded DNA molecule of approximately 36000 base pairs. The adenovirus DNA contains identical Inverted Terminal Repeats (ITR) of approximately 90-140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the genome ends.

Most adenoviral vectors currently used in gene therapy have a deletion in the E1 region, where novel genetic information can be introduced. The E1 deletion renders the recombinant virus replication defective (Levrero et al., 1991). It has been demonstrated extensively that recombinant adenovirus, in particular serotype 5 is suitable for efficient transfer of genes *in vivo* to the liver, the airway epithelium and solid tumors in animal models and human xenografts in immunodeficient mice (Bout, 1996; Blaese *et al.*, 1995). Thus, preferred methods for *in vivo* gene transfer into target cells make use of adenoviral vectors as gene delivery vehicles.

At present, six different subgroups of human adenoviruses have been proposed which in total encompasses 51 distinct adenovirus serotypes (see table 1). Besides these human adenoviruses an extensive number of animal adenoviruses have been identified (see Ishibashi et al, 1983).

A serotype is defined on the basis of its immunological distinctiveness as determined by quantitative neutralization with animal antisera (horse, rabbit). If neutralization shows a certain degree of cross-reaction between two viruses, distinctiveness of serotype is assumed if A) the hemagglutinins are unrelated, as shown by lack of cross-reaction on hemagglutination-inhibition, or B) substantial biophysical/ biochemical differences in DNA exist (Francki et al, 1991). The nine serotypes identified last (42-51) were isolated for the first time from HIV- infected patients (Hierholzer et al 1988; Schnurr et al 1993; De Jong et al 1998). For reasons not well understood, most of such immuno-compromised patients shed adenoviruses that were rarely or never isolated from immuno-competent individuals (Hierholzer et al 1988, 1992; Khoo et al, 1995, De Jong et al, 1998).

Besides differences towards the sensitivity against neutralizing antibodies of different adenovirus serotypes, it has also been shown that adenoviruses in subgroup C such as Ad2, and Ad5 bind to different receptors as compared to adenoviruses from subgroup B such as Ad3 (Defer et al, 1990). Likewise, it was demonstrated that receptor specificity could be altered by exchanging the Ad3 with the Ad 5 knob protein, and vice versa (Krasnykh et al, 1996; Stevenson et al, 1995, 1997). The adenovirus serotype 5 ("Ad5") is most widely used for gene therapy purposes. Similar to serotypes 2, 4 and 7, serotype 5 has a natural affiliation towards lung epithelia and other respiratory tissues. In contrast, it is known that, for instance, serotypes 40 and 41 have a natural affiliation towards the gastrointestinal tract. For a detailed overview of the disease association of the different adenovirus serotypes see table 2. The serotypes described above, differ in at least capsid proteins (penton-base, hexon), proteins responsible for

cell binding (fiber protein), and proteins involved in adenovirus replication.

One of the major problems of adenovirus gene therapy is thus that none of the above described serotypes are ideally suitable for delivering additional genetic material to host cells. Some have a somewhat limited host range, but have the benefit of being less immunogenic, some are the other way round. Some have a problem of being of a limited virulence, but have a broad host range and/or a reduced immunogenicity. To make things even more complicated this variation in the adenovirus serotypes is also very dependent on the host to be treated. Some hosts may already have encountered certain serotypes and thus mount a strong immune response to the serotype or a related serotype. Persons skilled in the art know that there are many other variations on this same theme.

Description of the Invention

The present invention now makes use of the fact that some adenoviruses have lower immunogenicity than others, which others typically excel in one of the other requirements for an efficient gene therapy regime, such as having a high specificity for a certain group of host cells, a good replication machinery in such host cells, a high rate of infection in certain host cells, etc.

The invention thus provides chimeric adenoviruses having the useful properties of at least two adenoviruses of different serotypes. Typically, more than two requirements from the previously given non-exhaustive list are required to obtain an adenovirus capable of efficiently transferring additional material to a host cell and therefore the invention provides adenovirus derived vectors which can be used as cassettes to

insert different adenoviral genes from different adenoviral serotypes at the required sites for obtaining a vector capable of expressing a chimeric adenovirus, whereby Of course, also a gene of interest can be inserted at for instance the site of E1 of the original adenovirus from which the vector is derived. In this manner the chimeric adenovirus to be produced can be adapted to the requirements and needs of certain hosts in need of gene therapy for certain disorders. Of course, to enable this production a packaging cell will generally be needed in order to produce sufficient amount of safe chimeric adenoviruses.

Thus, in one embodiment, the invention provides a chimeric adenovirus including at least a part of a fiber protein and/or a protein involved in replication of an adenovirus serotype providing the chimeric virus with a desired host range and/or improved replication properties and at least a part of a penton or hexon protein from another less antigenic adenovirus serotype resulting in a less antigenic chimeric adenovirus. Typically, such a virus will be produced using a vector (typically a plasmid, a cosmid or baculovirus system) which vector is Of course, also part of the present invention. A preferred vector is a vector which can be used to make a chimeric recombinant virus specifically adapted to the host to be treated and the disorder to be treated. Such a vector is another embodiment of the present invention. Thus, the invention also provides a recombinant vector derived from an adenovirus including at least one ITR and a packaging signal, having an insertion site for a nucleic acid sequence of interest, and further having an insertion site for functionally inserting a gene encoding a penton and/or a hexon protein of a first serotype of adenovirus and having an

insertion site for a gene encoding a fiber protein of a second adenovirus of a different serotype, and/or an insertion site for a gene derived from a serotype having improved characteristics in the function carried out by that gene or its product. Typically, the invention provides cassettes which allow for the production of any desired chimeric adenovirus, be it only derived from two serotypes or as many as needed to obtain the desired characteristics, whereby it is not always necessary that all characteristics are the best when seen as single properties. It may not even be necessary, for instance, to always alter penton and/or hexon together with another part of adenovirus genes. Sometimes the immunogenicity needs not be altered together with other properties. However, it is preferred to use penton and/or hexon genes from less immunogenic adenovirus serotypes. An important feature of the present invention is the means to produce the chimeric virus. Typically, one does not want an adenovirus batch to be administered to the host cell which contains replication competent adenovirus, although this is not always true. In general therefor it is desired to omit a number of genes (but at least one) from the adenoviral genome on the vector encoding the chimeric virus and to supply these genes in the genome of the cell in which the vector is brought to produce chimeric adenovirus. Such a cell is usually called a packaging cell. The invention thus also provides a packaging cell for producing a chimaeric adenovirus according to the invention, including in trans all elements necessary for adenovirus production not present on the adenoviral vector according to the invention. Typically, vector and packaging cell have to be adapted to one another in that they have all the necessary elements, but that

they do not have overlapping elements which lead to replication competent virus by recombination.

Thus, the invention also provides a kit of parts including a packaging cell according to the invention and a recombinant vector according the invention whereby there is essentially no sequence overlap leading to recombination resulting in the production of replication competent adenovirus between the cell and the vector.

In order to be able to precisely adapt the viral vector and provide the chimaeric virus with the desired properties at will, it is preferred that a library of adenoviral genes is provided whereby the genes are located within restriction sites. Typically, it is preferred to have same kinds of genes of different serotypes within the same restriction sites and to have that same restriction site in the adenoviral vector used to produce the chimaeric virus. If all sites for different genes are unique, then a system to pick and choose from has been made. One can cut a penton gene from the desired serotype from the library and insert it at the same site in the vector. One can then use a different restriction enzyme to cut a replication gene from the bank of a different serotype using another restriction enzyme and insert that gene at the corresponding restriction site in the chimaeric vector. Thus, it is to be preferred to have a vector according to the invention where the insertion sites are different and preferably unique restriction sites. Preferably, this vector is combined with a library having the corresponding genes within the same restriction sites. Methods to use this library and the vector are within the skill in the art and are part of the present invention. Typically, such a method includes a number of restriction and ligation steps and expression of the

result in a packaging cell. Also, one can use a library from which the different desired adenoviral genes are obtained through homologous recombination or a combination of restriction and recombination. Thus, the invention provides a method for producing a chimaeric adenovirus having a desired host range and diminished antigenicity, including providing a vector according to the invention having the desired insertion sites, inserting into the vector at least a functional part of a penton or hexon protein derived from an adenovirus serotype having relatively low antigenicity, inserting at least a functional part of a fiber protein derived from an adenovirus serotype having the desired host range and transfecting the vector in a packaging cell according to the invention and allowing for production of chimaeric viral particles. Of course, other combinations of other viral genes originating from different serotypes can also be inserted as disclosed herein before.

An immunogenic response to adenovirus that typically occurs is the production of neutralizing antibodies by the host. This is typically a reason for selecting a penton, hexon and/or fiber of a less immunogenic serotype.

Of course, it may not be necessary to make chimaeric adenoviruses which have complete proteins from different serotypes. It is well within the skill of the art to produce chimaeric proteins, for instance in the case of fiber proteins it is very well possible to have the base of one serotype and the shaft and the knob from another serotype. In this manner it becomes possible to have the parts of the protein responsible for assembly of viral particles originate from one serotype, thereby enhancing the production of intact viral particles. Thus, the invention also provides a chimaeric

adenovirus according to the invention, wherein the hexon, penton and/or fiber proteins are chimaeric proteins originating from different adenovirus serotypes. Besides generating chimaeric adenoviruses by swapping entire wild type hexon, penton, fiber (protein) genes etc. or parts thereof, it is also within the scope of the present invention to insert hexon, penton, fiber (protein) genes etc. carrying mutations such as point mutations, deletions, insertions etc. which can be easily screened for preferred characteristics such as temperature stability, assembly, anchoring, redirected infection, altered immune response etc. Again other chimaeric combinations can also be produced and are within the scope of the present invention.

The availability of a library of nucleic acids derived from different serotypes allows, among others, the generation of a library of chimaeric adenoviruses. The invention therefore further provides a library of chimaeric adenoviruses. In one embodiment the invention provides a library of chimaeric adenoviruses wherein the adenoviruses include chimeric capsids, *i.e.*, including capsid proteins derived at least in part from at least two different adenovirus serotypes. Preferably, nucleic acid and/or protein or parts thereof, from at least one representative adenovirus of each adenovirus subgroup is represented in the (chimaeric) adenovirus library. Preferably, nucleic acid and/or protein or parts thereof is derived from more than one representative of each adenovirus subgroup. Most preferably, the library includes nucleic acid and/or protein or a part thereof, from essentially every known representative of each adenovirus subgroup. Nucleic acid and/or protein or parts thereof derived from more than one representative adenovirus from each adenovirus subgroup in the (chimaeric)

library is desired because a desirable property may not be a general property of a subgroup. Also, a desirable property of a subgroup of adenovirus may be expressed in different amounts on the various members of the subgroup. Ensuring that more than one representative of a subgroup is represented in the library thus warrants the selection of the best expressor of the desired property.

Typically, a library of chimaeric adenoviruses (or a part thereof) is used in screening assays to determine properties of the chimaeric adenoviruses. Any particular chimaeric adenovirus including particularly desirable properties can thereby be identified and subsequently be used in, for instance, the development of an improved nucleic acid delivery vehicle. Desirable properties the chimaeric adenovirus library may be screened for include, but are not limited to, target cell specificity, reduced immunogenicity, increased immunogenicity, re-directed neutralization, re-directed hemagglutination, improved infection efficiency, reduced toxicity, improved replication and/or improved pharmacokinetics such as altered tissue distribution upon *in vivo* administration. Comparison of properties of different chimaeric adenoviruses can lead to the delineation of adenovirus elements involved in providing an adenovirus with the property. Such knowledge can then be used to further optimize nucleic acid delivery vehicles. In one aspect the invention provides a selection of (chimaeric) adenoviruses with an improved capacity to transduce macrophage- or fibroblast-like cells compared to adenovirus 5, preferably the (chimaeric) adenoviruses include at least part of a tissue tropism determining part of a fiber protein of an adenovirus of subgroup B, or a derivative and/or analogue of the fiber protein.

The invention further provides a selection of (chimaeric) adenoviruses with an improved capacity to transduce smooth muscle cells compared to adenovirus 5, preferably the (chimaeric) adenoviruses include at least part of a tissue tropism determining part of a fiber protein of an adenovirus of subgroup B, or a derivative and/or analogue of the fiber protein. A chimaeric adenovirus library of the invention may further be used to study adenovirus biology. Such a library is for instance very well suited to study differences in the biology of the various adenovirus serotypes. In one aspect the invention provides a selection of (chimaeric) adenoviruses, capable of transducing a CAR negative cell. Preferably, the CAR negative cell is a amnion fluid cell or a derivative thereof. Preferably, the amnion fluid cell is a chorion villi cell or a derivative thereof. Preferably, the CAR negative cell is a CAR negative hemopoietic cell, such as but not limited to an erythroid precursor cell and/or a monocyte precursor cell and/or derivatives thereof. Preferably, the (chimeric) adenoviruses capable of transducing a CAR negative cell include at least an adenovirus receptor binding part of a fiber protein from an adenovirus of subgroup D or F.

In one aspect, the invention provides a chimaeric adenovirus including a re-directed neutralization pattern compared to Ad5. Re-directed neutralization is useful in a number of circumstances. For instance, but not limited to, getting round pre-existing neutralizing antibodies in a patient administered with the chimaeric adenovirus. Pre-existing neutralizing antibodies would neutralize the adenovirus and thereby diminish the effective amount of virus administered. This effect is usually not desired in for instance gene therapy settings wherein a nucleic acid is to be delivered to target

cells. However, pre-existing neutralizing antibodies can for instance in other gene therapy applications be an advantage when the nucleic acid of interest delivered through the chimaeric adenovirus should not be delivered to cells throughout the body. Local delivery for instance by using a needle in a solid tissue combined with the presence of neutralizing antibodies in the blood that can neutralize leaking chimaeric adenovirus can in that case help to contain the transduction to a certain area.

In another aspect, the invention provides a chimaeric adenovirus including a re-directed hemagglutination pattern compared to adenovirus 5. Re-directed hemagglutination is useful in a number of circumstances. Hemagglutinated material is preferentially taken up by macrophages and derivatives and/or precursors. Thus, enhanced hemagglutination of a chimaeric adenovirus is preferred in case where enhanced delivery of nucleic acid to the macrophages is desired. However, in general the target cell will not be the macrophages thus in those cases a reduced hemagglutination is desired. A chimaeric adenovirus re-directed in its hemagglutination is useful for many applications which the person skilled art can now think of and thus form an integral part of the present invention.

Brief Description of the Figures

FIG. 1 schematically presents adapter plasmid pMLPI.TK.

FIG. 2 schematically presents adapter plasmid pAd/L420-HSA.

FIG. 3 schematically presents adapter plasmid pAd5/CLIP

FIG. 4 schematically presents a two plasmid system for the generation of recombinant adenoviruses.

FIG. 5 schematically presents a three plasmid system for the generation of recombinant adenoviruses.

FIG. 6 schematically presents the generation of plasmid pBr/AdBamRDeltaFib in which part of the Adenovirus type 5 fiber DNA is replaced by a short DNA stretch containing a unique NsiI site. (Primer Sequences Correspond to SEQ ID NOS: 56-57)

FIG. 7 depicts the fiber protein sequences of adenovirus serotypes 8, 9, 13, 14,20, 23, 24, 25, 27, 28, 29, 30, 32, 33, 34, 35, 36, 37, 38, 39, 42, 43, 44, 45, 46, 47, 48, 49, and 51. (SEQ ID NOS: 14-46) Bold letters represent part of the tail of Ad5. If bold letters not present, it means that a PCR fragment was sequenced which did not contain the Ad5 tail. An X, present in the sequence means unidentified amino acid due to unidentified nucleotide. At the end of the sequence the stop codon of the fiber is presented by a dot.

FIG. 8 compares the *in vivo* bio-distribution of I^{123} labeled Ad5 and an adenovirus chimeric for fiber protein. Radio-labeled adenovirus (10^{10} virus particles, 0.1-2 MBq) was intravenously administered into the tail vein. As a control, a similar amount of free label was injected into the control animal. Rats were sacrificed after one hour and organs calibrated. Radioactivity of the in the figure indicated organs was measured with a scintillation counter and is expressed as counts per minute per gram tissue.

FIG. 9 schematically presents the generation of plasmid pBr/Ad.Eco-PmeΔHexon. Also shown is the sequence of the oligonucleotides delta hex 1-4 used to delete the DNA encoding for the hexon of Ad5 protein.

FIG. 10 depicts the hexon protein sequences of adenovirus serotypes 34, 35, 36, and 41. An X, present in the sequence means unidentified amino acid due to unidentified nucleotide.

At the end of the sequence the stop codon of the hexon is presented by a dot.

Detailed Description of the Invention

It has been demonstrated in mice that upon *in vivo* systemic delivery of recombinant Ad5 for gene therapy purposes approximately 99% of the virus is trapped in the liver (Herz et al, 1993). Therefore, alteration of the Ad5 host cell range to be able to target other organs *in vivo* is a major interest of the invention, particularly in combination with other alterations, in particular the immunogenicity.

The initial step for successful infection is binding of adenovirus to its target cell, a process mediated through fiber protein. The fiber protein has a trimeric structure (Stouten et al, 1992) with different lengths depending on the virus serotype (Signas et al 1985; Kidd et all 1993). Different serotypes have polypeptides with structurally similar N and C termini, but different middle stem regions. N-terminally, the first 30 amino acids are involved in anchoring of the fiber to the penton base (Chroboczek et al, 1995), especially the conserved FNPVYP region in the tail (Arnberg et al 1997). The C-terminus, or knob, is responsible for initial interaction with the cellular adenovirus receptor. After this initial binding secondary binding between the capsid penton base and cell-surface integrins leads to internalization of viral particles in coated pits and endocytosis (Morgan et al, 1969; Svensson et al, 1984; Varga et al, 1992; Greber et al, 1993; Wickham et al, 1994). Integrins are αβ-heterodimers of which at least 14 α-subunits and 8 β-subunits have been identified

(Hynes et al, 1992). The array of integrins expressed in cells is complex and will vary between cell types and cellular environment. Although the knob contains some conserved regions, between serotypes, knob proteins show a high degree of variability, indicating that different adenovirus receptors exist. For instance, it has been demonstrated that adenoviruses of subgroup C (Ad2, Ad5) and adenoviruses of subgroup B (Ad3) bind to different receptors (Defner et al, 1990). The fiber protein also contains the type specific γ-antigen, which together with the ε-antigen of the hexon determines the serotype specificity. The γ-antigen is localized on the fiber and it is known that it consists of 17 amino acids (Eiz et al, 1997). The anti-fiber antibodies of the host are therefore directed to the trimeric structure of the knob. The anti-fiber antibodies together with antibodies directed against the penton base and hexon proteins are responsible for the neutralization of adenovirus particles. First, the anti-fiber antibodies uncoat the adenovirus particles after which the penton base is accessible to the anti-penton base antibodies (Gahery-Segard et al, 1998). Although this seems to be a very effective way to neutralize adenovirus particles others have described that the anti-hexon antibodies are the most effective ones in neutralization of the particles (Gall et al, 1996).

To obtain re-directed infection of recombinant Ad5, several approaches have been or still are under investigation. Wickham et al has altered the RGD (Arg, Gly, Asp) motif in the penton base which is believed to be responsible for the $\alpha\alpha_V\beta\beta_3$ and $\alpha_V\beta_5$ integrin binding to the penton base. They have replaced this RGD motif by another peptide motif which is specific for the $\alpha_4\beta_1$ receptor. In this way, targeting the

adenovirus to a specific target cell could be accomplished (Wickham et al, 1995, 1996). Krasnykh et al has made use of the HI loop available in the knob. This loop is, based on X-ray crystallographics, located on the outside of the knob trimeric structure and therefore is thought not to contribute to the intramolecular interactions in the knob (Krasnykh et al, 1998). However, complete CAR independent infection was not observed.

The present invention provides a method and means by which adenoviruses can be constructed with an altered immune response, or with the absence or decreased infection in antigen presenting cells such as dendritic cells or macrophages. The present invention further provides methods for the generation of chimaeric adenoviruses as described herein which can be targeted to specific cell types *in vitro* as well as *in vivo* have an altered tropism for certain cell types. The present invention further provides a method and means by which such an adenovirus can be used as a protein or nucleic acid delivery vehicle to a specific cell type or tissue.

The generation of chimaeric adenoviruses based on Ad5 with modified late genes is described. For this purpose, three plasmids, which together contain the complete Ad5 genome, were constructed. From these plasmids, the DNA encoding the Ad5 penton-base protein, hexon protein, and fiber protein were removed and replaced by linker DNA sequences which facilitate easy cloning. These plasmids subsequently served as template for the insertion of DNA encoding for penton-base protein, hexon protein, and fiber protein derived from different adenovirus, serotypes (human or animal). The DNAs derived from the different serotypes were obtained using the polymerase chain reaction technique in combination with (degenerate) oligonucleotides. At the former E1 location in the genome of

Ad5, any gene of interest can be cloned. A single transfection procedure of the three plasmids together resulted in the formation of a recombinant chimaeric adenovirus. This new technology of libraries consisting of chimaeric adenoviruses thus allows for a rapid screening for improved recombinant adenoviral vectors for *in vitro* and *in vivo* gene therapy purposes.

Although successful introduction of changes in the Ad5 fiber and penton-base have been reported, the complex structure of knob and the limited knowledge of the precise amino acids interacting with CAR render such targeting approaches laborious and difficult.

To overcome the limitations described previously, we used pre-existing adenovirus fibers, penton base proteins, and hexon proteins derived from other adenovirus serotypes. By generating chimaeric Ad5 libraries containing structural proteins of alternative adenovirus serotypes, we have developed a technology which enables rapid screening for a recombinant adenoviral vector with preferred characteristics.

In one aspect, this invention describes the use of chimaeric adenoviruses to overcome, natural existing or induced, neutralizing host activity towards recombinant adenoviruses administered *in vivo* for therapeutic applications. The host immune response is predominantly directed against penton base - and hexon proteins present in the adenoviral capsid and to a lesser extend directed to fiber.

The adenovirus serotypes are defined by the inability to cross-react with neutralizing antibodies in animal sera. Therefore chimaeric viruses based on for example Ad5 but chimaeric for penton base protein, and/ or hexon protein provoke an altered, less severe immune response. The need for such chimaeric adenoviruses is stressed by observations that

1) repeated systemic delivery of recombinant Ad5 is unsuccessful due to formation of high titers of neutralizing antibodies against the recombinant Ad5 (Schulick et al, 1997), and 2) pre-existing or natural immunity.

This aspect of the invention therefore circumvents the inability to repeat the administration of an adenovirus for gene therapy purposes. Preferably, the penton base-, hexon-, and fiber proteins are derived from adenoviruses in subgroup B and D and are more specifically of the adenovirus serotype 16, 24, 33, 36, 38, 39, 42, and 50. This latter is because these serotypes are rarely isolated from humans indicating that low titers of circulating neutralizing antibodies are present against these serotypes.

In another aspect, this invention describes chimaeric adenoviruses and methods to generate these viruses that have an altered tropism different from that of Ad5. For example, viruses based on Ad5 but displaying any adenovirus fiber existing in nature. This chimaeric Ad5 is able to infect certain cell types more efficiently, or less efficiently *in vitro* and *in vivo* than the Ad5. Such cells include but are not limited to endothelial cells, smooth muscle cells, dendritic cells, neuronal cells, glial cells, synovical cells, lung epithelial cells, hemopoietic stem cells, monocytic/ macrophage cells etc.

In another aspect, this invention describes methods which identify chimaeric adenoviruses that display improved *in vitro* amplification in static or suspension cell cultures. Adenoviruses derived from different subgroups, but also within one subgroup, display a high variability in productive infection in cell types that are used for production of recombinant adenovirus. Table 2 lists an overview of different adenovirus serotypes and their association with human disease, demonstrating that replication of a given adenovirus serotype is enhanced in certain cell types. For the production of

recombinant adenoviruses for gene therapy purposes, several cell lines are available. These include but do not limit PER.C6, 911, 293, and E1 A549. These adenovirus producer cells may not be the most suited cell types to amplify Ad5 based viruses. Therefore, in this aspect of the invention we select adenoviruses from different serotypes based on their ability to propagate for example on PER.C6 and use their early genes (without E1) and ITRs to construct chimaeric viruses which are superior in terms of propagation and thus yield higher titers as compared to commonly used adenovirus serotype 2 or 5.

In another aspect, the invention describes the construction and use of libraries consisting of distinct parts of Ad5 in which one or more genes or sequences have been replaced with DNA derived from alternative human or animal serotypes. This set of constructs, in total encompassing the complete adenovirus genome, allows for the construction of unique chimaeric adenoviruses customized for a certain group of patients or even a single individual.

The chimeric adenoviruses may, but need not, contain deletions in the E1 region and insertions of heterologous genes linked either or not to a promoter. Furthermore, chimaeric adenoviruses may, or may not, contain deletions in the E3 region and insertions of heterologous genes linked to a promoter. Furthermore, chimaeric adenoviruses may, or may not, contain deletions in the E2 and/ or E4 region and insertions of heterologous genes linked to a promoter. In the latter case E2 and/ or E4 complementing cell lines are required to generated recombinant adenoviruses.

The invention is further explained with the help of the following illustrative Examples.

Examples

Example 1: Generation of Ad5 genomic plasmid clones

The complete genome of Ad5 has been cloned into various plasmids or cosmids to allow easy modification of parts of the Ad5 genome, while still retaining the capability to produce recombinant virus. For this purpose the following plasmids were generated:

1. pBr/Ad.Bam-rITR (ECACC deposit P97082122)

In order to facilitate blunt end cloning of the ITR sequences, wild-type human adenovirus type 5 (Ad5) DNA was treated with Klenow enzyme in the presence of excess dNTPs. After inactivation of the Klenow enzyme and purification by phenol/chloroform extraction followed by ethanol precipitation, the DNA was digested with BamHI. This DNA preparation was used without further purification in a ligation reaction with pBr322 derived vector DNA prepared as follows: pBr322 DNA was digested with EcoRV and BamHI, dephosphorylated by treatment with TSAP enzyme (Life Technologies) and purified on LMP agarose gel (SeaPlaque GTG). After transformation into competent *E.coli* DH5a (Life Techn.) and analysis of ampiciline resistant colonies, one clone was selected that showed a digestion pattern as expected for an insert extending from the BamHI site in Ad5 to the right ITR.

Sequence analysis of the cloning border at the right ITR revealed that the most 3' G residue of the ITR was missing, the remainder of the ITR was found to be correct. The missing G residue is complemented by the other ITR during replication.

2. pBr/Ad.Sal-rITR (ECACC deposit P97082119)

pBr/Ad.Bam-rITR was digested with BamHI and SalI. The vector fragment including the adenovirus insert was isolated in LMP agarose (SeaPlaque GTG) and ligated to a 4.8 kb SalI-BamHI fragment obtained from wt Ad5 DNA and purified with the Geneclean II kit (Bio 101, Inc.). One clone was chosen and the integrity of the Ad5 sequences was determined by restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains adeno type 5 sequences from the SalI site at bp 16746 up to and including the rITR (missing the most 3' G residue).

3. pBr/Ad.Cla-Bam (ECACC deposit P97082117)

wt Adeno type 5 DNA was digested with ClaI and BamHI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBr322 was digested with the same enzymes and purified from agarose gel by Geneclean. Both fragments were ligated and transformed into competent DH5a. The resulting clone pBr/Ad.Cla-Bam was analyzed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

4. pBr/Ad.AflII-Bam (ECACC deposit P97082114)

Clone pBr/Ad.Cla-Bam was linearized with EcoRI (in pBr322) and partially digested with AflII. After heat inactivation of AflII for 20' at 65°C the fragment ends were filled in with Klenow enzyme. The DNA was then ligated to a blunt double stranded oligo linker containing a PacI site (5'-AATTGTCTTAATTAACCGCTTAA-3') (SEQ. ID. NO. 47) ~~(SEQ. ID. NO. __)~~. This linker was made by annealing the following two oligonucleotides: 5'-AATTGTCTTAATTAACCGC-3' (SEQ ID NO. 84) ~~(SEQ. ID. NO. __)~~ and 5'-AATTGCGGTTAATTAAGAC-3' (SEQ. ID. NO. 85) ~~(SEQ. ID. NO. __)~~, followed by blunting

with Klenow enzyme. After precipitation of the ligated DNA to change buffer, the ligations were digested with an excess PacI enzyme to remove concatamers of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences, was isolated in LMP agarose (SeaPlaque GTG), religated and transformed into competent DH5a. One clone that was found to contain the PacI site and that had retained the large adeno fragment was selected and sequenced at the 5' end to verify correct insertion of the PacI linker in the (lost) AflII site.

5. pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and pBr/Ad.Bam-rITR#8 (ECACC deposit P97082121)

To allow insertion of a PacI site near the ITR of Ad5 in clone pBr/Ad.Bam-rITR, about 190 nucleotides were removed between the ClaI site in the pBr322 backbone and the start of the ITR sequences. This was done as follows: pBr/Ad.Bam-rITR was digested with ClaI and treated with nuclease Bal31 for varying lengths of time (2', 5', 10' and 15'). The extent of nucleotide removal was followed by separate reactions on pBr322 DNA (also digested at the ClaI site), using identical buffers and conditions. Bal31 enzyme was inactivated by incubation at 75°C for 10 minutes, the DNA was precipitated and resuspended in a smaller volume of TE buffer. To ensure blunt ends, DNAs were further treated with T4 DNA polymerase in the presence of excess dNTPs. After digestion of the (control) pBr322 DNA with SalI, satisfactory degradation (~150 bp) was observed in the samples treated for 10' or 15'. The 10' or 15' treated pBr/Ad.Bam-rITR samples were then ligated to the above described blunted PacI linkers (See pBr/Ad.AflII-Bam). Ligations were purified by precipitation, digested with excess

PacI and separated from the linkers on an LMP agarose gel. After religation, DNAs were transformed into competent DH5a and colonies analyzed. Ten clones were selected that showed a deletion of approximately the desired length and these were further analyzed by T-track sequencing (T7 sequencing kit, Pharmacia Biotech). Two clones were found with the PacI linker inserted just downstream of the rITR. After digestion with PacI, clone #2 has 28 bp and clone #8 has 27 bp attached to the ITR.

6. pWE/Ad.AflII-rITR (ECACC deposit P97082116)

Cosmid vector pWE15 (Clontech) was used to clone larger Ad5 inserts. First, a linker containing a unique PacI site was inserted in the EcoRI sites of pWE15 creating pWE.pac. To this end, the double stranded PacI oligo as described for pBr/Ad.AflII-BamHI was used but now with its EcoRI protruding ends. The following fragments were then isolated by electro-elution from agarose gel: pWE.pac digested with PacI, pBr/AflII-Bam digested with PacI and BamHI and pBr/Ad.Bam-rITR#2 digested with BamHI and PacI. These fragments were ligated together and packaged using l phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into host bacteria, colonies were grown on plates and analyzed for presence of the complete insert. pWE/Ad.AflII-rITR contains all adenovirus type 5 sequences from bp 3534 (AflII site) up to and including the right ITR (missing the most 3' G residue).

7. pBr/Ad.lITR-Sal(9.4) (ECACC deposit P97082115)

Adeno 5 wt DNA was treated with Klenow enzyme in the presence of excess dNTPs and subsequently digested with SalI.

Two of the resulting fragments, designated left ITR-Sal(9.4) and Sal(16.7)-right ITR, respectively, were isolated in LMP agarose (Seaplaque GTG). pBr322 DNA was digested with EcoRV and SalI and treated with phosphatase (Life Technologies). The vector fragment was isolated using the Geneclean method (BIO 101, Inc.) and ligated to the Ad5 SalI fragments. Only the ligation with the 9.4 kb fragment gave colonies with an insert. After analysis and sequencing of the cloning border a clone was chosen that contained the full ITR sequence and extended to the SalI site at bp 9462.

8. pBr/Ad.lITR-Sal(16.7) (ECACC deposit P97082118)

pBr/Ad.lITR-Sal(9.4) is digested with SalI and dephosphorylated (TSAP, Life Technologies). To extend this clone upto the third SalI site in Ad5, pBr/Ad.Cla-Bam was linearized with BamHI and partially digested with SalI. A 7.3 kb SalI fragment containing adenovirus sequences from 9462-16746 was isolated in LMP agarose gel and ligated to the SalI-digested pBr/Ad.lITR-Sal(9.4) vector fragment.

9. pWE/Ad.AflII-EcoRI

pWE.pac was digested with ClaI and 5' protruding ends were filled using Klenow enzyme. The DNA was then digested with PacI and isolated from agarose gel. pWE/AflII-rITR was digested with EcoRI and after treatment with Klenow enzyme digested with PacI. The large 24 kb fragment containing the adenoviral sequences was isolated from agarose gel and ligated to the ClaI-digested and blunted pWE.pac vector using the Ligation Express^{tm} kit from Clontech. After transformation of Ultracompetent XL10-Gold cells from Stratagene, clones were

identified that contained the expected insert. pWE/AflII-EcoRI contains Ad5 sequences from bp 3534-27336.

10. Construction of new adapter plasmids

The absence of sequence overlap between the recombinant adenovirus and E1 sequences in the packaging cell line is essential for safe, RCA-free generation and propagation of new recombinant viruses. The adapter plasmid pMLPI.TK (FIG. 1) is an example of an adapter plasmid designed for use according to the invention in combination with the improved packaging cell lines of the invention. This plasmid was used as the starting material to make a new vector in which nucleic acid molecules including specific promoter and gene sequences can be easily exchanged.

First, a PCR fragment was generated from pZipΔΔMo+PyF101(N^-) template DNA (described in PCT/NL96/00195) with the following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 48) and LTR-2: 5'-GCG GAT CCT TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3' ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 49). Pwo DNA polymerase (Boehringer Mannheim) was used according to manufacturers protocol with the following temperature cycles: once 5' at 95°C; 3' at 55°C; and 1' at 72°C, and 30 cycles of 1' at 95°C, 1' at 60°C, 1' at 72°C, followed by once 10' at 72°C. The PCR product was then digested with BamHI and ligated into pMLP10 (Levrero *et al.*, 1991) vector digested with PvuII and BamHI, thereby generating vector pLTR10. This vector contains adenoviral sequences from bp 1 up to bp 454 followed by a promoter consisting of a part of the Mo-MuLV LTR having its wild-type enhancer sequences replaced by the enhancer from a mutant polyoma virus (PyF101). The promoter fragment was

designated L420. Next, the coding region of the murine HSA gene was inserted. pLTR10 was digested with BstBI followed by Klenow treatment and digestion with NcoI. The HSA gene was obtained by PCR amplification on pUC18-HSA (Kay *et al.*, 1990) using the following primers: HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA TGG TGG C-3' (SEQ ID. NO. 50) and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT CTA CTA ACA GTA GAG ATG TAG AA-3' (SEQ ID NO. 51). The 269 bp amplified fragment was subcloned in a shuttle vector using the NcoI and BglII sites. Sequencing confirmed incorporation of the correct coding sequence of the HSA gene, but with an extra TAG insertion directly following the TAG stop codon. The coding region of the HSA gene, including the TAG duplication was then excised as a NcoI (sticky)-SalI (blunt) fragment and cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt) fragment from pLTR10, resulting in pLTR-HSA10.

Finally, pLTR-HSA10 was digested with EcoRI and BamHI after which the fragment containing the left ITR, packaging signal, L420 promoter and HSA gene was inserted into vector pMLPI.TK digested with the same enzymes and thereby replacing the promoter and gene sequences. This resulted in the new adapter plasmid pAd/L420-HSA (FIG. 2) that contains convenient recognition sites for various restriction enzymes around the promoter and gene sequences. SnaBI and AvrII can be combined with HpaI, NheI, KpnI, HindIII to exchange promoter sequences, while the latter sites can be combined with the ClaI or BamHI sites 3' from HSA coding region to replace genes in this construct.

Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the promoter, gene and poly A sequences in pAd/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a poly-A

signal. For this purpose, pAd/L420-HSA was digested with AvrII and BglII followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pcDNA1/amp (Invitrogen) obtained by digestion with HhaI and AvrII followed by treatment with T4 DNA polymerase. This adapter plasmid was named pCLIP.Luc (FIG. 3).

11. Generation of recombinant adenoviruses

To generate E1-deleted recombinant adenoviruses with the new plasmid-based system, the following constructs are prepared:

a) An adapter construct containing the expression cassette with the gene of interest linearized with a restriction enzyme that cuts at the 3' side of the overlapping adenoviral genome fragment, preferably not containing any pBr322 vector sequences, and

b) A complementing adenoviral genome construct pWE/Ad.AflII-rITR digested with PacI.

These two DNA molecules are further purified by phenol/chloroform extraction and EtOH precipitation. Co-transfection of these plasmids into an adenovirus packaging cell line, preferably a cell line according to the invention, generates recombinant replication deficient adenoviruses by a one-step homologous recombination between the adapter and the complementing construct (FIG. 4).

Alternatively, instead of pWE/Ad.AflII-rITR, other fragments can be used, *e.g.*, pBr/Ad.Cla-Bam digested with EcoRI and BamHI or pBr/Ad.AflII-BamHI digested with PacI and BamHI can be combined with pBr/Ad.Sal-rITR digested with SalI. In this case, three plasmids are combined and two homologous

recombinations are needed to obtain a recombinant adenovirus (FIG. 5). It is to be understood that those skilled in the art may use other combinations of adapter and complementing plasmids without departing from the present invention.

A general protocol as outlined below and meant as a non-limiting example of the present invention has been performed to produce several recombinant adenoviruses using various adapter plasmids and the Ad.AflII-rITR fragment. Adenovirus packaging cells (PER.C6) were seeded in ~25 cm^2 flasks and the next day when they were at ~80% confluency, transfected with a mixture of DNA and lipofectamine agent (Life Techn.) as described by the manufacturer. Routinely, 40 μl lipofectamine, 4 μg adapter plasmid and 4 μg of the complementing adenovirus genome fragment AflII- rITR (or 2 μg of all three plasmids for the double homologous recombination) are used. Under these conditions transient transfection efficiencies of ~50% (48 hrs post transfection) are obtained as determined with control transfections using a pAd/CMV-LacZ adapter. Two days later, cells are passaged to ~80 cm^2 flasks and further cultured. Approximately five (for the single homologous recombination) to eleven days (for the double homologous recombination) later a cytopathogenic effect (CPE) is seen, indicating that functional adenovirus has formed. Cells and medium are harvested upon full CPE and recombinant virus is released by freeze-thawing. An extra amplification step in an 80 cm^2 flask is routinely performed to increase the yield since at the initial stage the titers are found to be variable despite the occurrence of full CPE. After amplification, viruses are harvested and plaque purified on PER.C6 cells. Individual plaques are tested for viruses with active transgenes.

Besides replacements in the E1 region, it is possible to delete or replace (part of) the E3 region in the adenovirus because E3 functions are not necessary for the replication, packaging and infection of the (recombinant) virus. This creates the opportunity to use a larger insert or to insert more than one gene without exceeding the maximum package size (approximately 105% of wt genome length). This can be done, *e.g.*, by deleting part of the E3 region in the pBr/Ad.Bam-rITR clone by digestion with XbaI and religation. This removes Ad5 wt sequences 28592-30470 including all known E3 coding regions. Another example is the precise replacement of the coding region of gp19K in the E3 region with a polylinker allowing insertion of new sequences. This, 1) leaves all other coding regions intact and 2) obviates the need for a heterologous promoter since the transgene is driven by the E3 promoter and pA sequences, leaving more space for coding sequences.

To this end, the 2.7 kb EcoRI fragment from wt Ad5 containing the 5' part of the E3 region was cloned into the EcoRI site of pBluescript (KS$^-$) (Stratagene). Next, the HindIII site in the polylinker was removed by digestion with EcoRV and HincII and subsequent religation. The resulting clone pBS.Eco-Eco/ad5DHIII was used to delete the gp19K coding region. Primers 1 (5'-GGG TAT TAG GCC AA AGG CGC A-3') ~~(SEQ. ID. NO.)~~ (SEQ. ID. NO 52) and 2 (5'-GAT CCC ATG GAA GCT TGG GTG GCG ACC CCA GCG-3') ~~(SEQ. ID. NO.)~~ (SEQ. ID. NO 53) were used to amplify a sequence from pBS.Eco-Eco/Ad5DHIII corresponding to sequences 28511 to 28734 in wt Ad5 DNA. Primers 3 (5'-GAT CCC ATG GGG ATC CTT TAC TAA GTT ACA AAG CTA-3') ~~(SEQ. ID. NO.)~~ (SEQ. ID. NO. 54) and 4 (5'-GTC GCT GTA GTT GGA CTG G-3') ~~(SEQ. ID. NO.)~~ (SEQ ID NO: 55) were used on the same DNA to amplify Ad5 sequences from 29217 to 29476. The two resulting PCR fragments were ligated together by virtue of the new introduced

NcoI site and subsequently digested with XbaI and MunI. This fragment was then ligated into the pBS.Eco-Eco/ad5ΔΔHIII vector that was digested with XbaI (partially) and MunI generating pBS.Eco-Eco/ad5ΔHIII.Δgp19K. To allow insertion of foreign genes into the HindIII and BamHI site, an XbaI deletion was made in pBS.Eco-Eco/ad5ΔHIII.Δgp19K to remove the BamHI site in the Bluescript polylinker. The resulting plasmid pBS.Eco-Eco/ad5ΔHIIIΔgp19KΔΔXbaI, contains unique HindIII and BamHI sites corresponding to sequences 28733 (HindIII) and 29218 (BamHI) in Ad5. After introduction of a foreign gene into these sites, either the deleted XbaI fragment is re-introduced, or the insert is recloned into pBS.Eco-Eco/ad5ΔHIII.Δgp19K using HindIII and for example MunI. Using this procedure, we have generated plasmids expressing HSV-TK, hIL-1a, rat IL-3, luciferase or LacZ. The unique SrfI and NotI sites in the pBS.Eco-Eco/ad5ΔHIII.Δgp19K plasmid (with or without inserted gene of interest) are used to transfer the region including the gene of interest into the corresponding region of pBr/Ad.Bam-rITR, yielding construct pBr/Ad.Bam-rITRΔΔgp19K (with or without inserted gene of interest). This construct is used as described *supra* to produce recombinant adenoviruses. In the viral context, expression of inserted genes is driven by the adenovirus E3 promoter.

Recombinant viruses that are both E1- and E3- deleted are generated by a double homologous recombination procedure as described above for E1-replacement vectors using a plasmid-based system consisting of:

a) an adapter plasmid for E1 replacement according to the invention, with or without insertion of a first gene of interest,

b) the pWE/Ad.AflII-EcoRI fragment, and

c) the pBr/Ad.Bam-rITRΔgp19K plasmid with or without insertion of a second gene of interest.

In addition to manipulations in the E3 region, changes of (parts of) the E4 region can be accomplished easily in pBr/Ad.Bam-rITR. Generation and propagation of such a virus, however, in some cases demands complementation *in trans*.

Example 2: Generation of Ad5 based viruses with chimaeric fiber proteins

The method described *infra* to generate recombinant adenoviruses by co-transfection of two, or more separate cloned adenoviral sequences. These cloned adenoviral sequences were subsequently used to remove specific Ad5 sequences in order to generate template clones which allow for the easy introduction of DNA sequences derived from other adenovirus serotypes. As an example of these template clones, the construction of plasmids enabling swapping of DNA encoding for fiber protein is given below.

Generation of adenovirus template clones lacking DNA encoding fiber

The fiber coding sequence of Ad5 is located between nucleotides 31042 and 32787. To remove the Ad5 DNA encoding fiber we started with construct pBr/Ad.Bam-rITR. First, a NdeI site was removed from this construct. For this purpose, pBr322 plasmid DNA was digested with NdeI after which protruding ends were filled using Klenow enzyme. This pBr322 plasmid was then re-ligated, digested with NdeI and transformed into *E.coli* DH5αα. The obtained pBr/ΔΔNdeI plasmid was digested with ScaI

and SalI and the resulting 3198 bp vector fragment was ligated to the 15349 bp ScaI-SalI fragment derived from pBr/Ad.BamrITR, resulting in plasmid pBr/Ad.Bam-rITRΔΔNdeI which hence contained a unique NdeI site. Next a PCR was performed with oligonucleotides NY-up: 5'- CGA **CAT ATG** TAG ATG CAT TAG TTT GTG TTA TGT TTC AAC GTG-3' (SEQ. ID. NO. 56) and NY-down: 5'-GGA GAC CAC TGC CAT GTT-3' (SEQ. ID. NO. 57) (FIG. 6). During amplification, both a NdeI (bold face) and a NsiI restriction site (underlined) were introduced to facilitate cloning of the amplified fiber DNAs. Amplification consisted of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45 sec. at 72°C. The PCR reaction contained 25 pmol of oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with 1.5 mM $MgCl_2$, and 1 unit of Elongase heat stable polymerase (Gibco, The Netherlands). One-tenth of the PCR product was run on an agarose gel which demonstrated that the expected DNA fragment of ± 2200 bp was amplified. This PCR fragment was subsequently purified using Geneclean kit system (Bio101 Inc.). Then, both the construct pBr/Ad.Bam-rITRΔNdeI as well as the PCR product were digested with restriction enzymes NdeI and SbfI. The PCR fragment was subsequently cloned using T4 ligase enzyme into the NdeI and SbfI digested pBr/Ad.Bam-rITRΔNdeI, generating pBr/Ad.BamRΔFib. This plasmid allows insertion of any PCR amplified fiber sequence through the unique NdeI and NsiI sites that are inserted in place of the removed fiber sequence. Viruses can be generated by a double homologous recombination in packaging cells described *infra* using an adapter plasmid, construct pBr/Ad.AflII-EcoRI digested with PacI and EcoRI and a pBr/Ad.BamRΔFib construct in which heterologous fiber sequences have been inserted. To increase the efficiency of

virus generation, the construct pBr/Ad.BamRΔFib was modified to generate a PacI site flanking the right ITR. Hereto, pBr/Ad.BamRΔFib was digested with AvrII and the 5 kb adeno fragment was isolated and introduced into the vector pBr/Ad.Bam-rITR.pac#8 replacing the corresponding AvrII fragment. The resulting construct was named pBr/Ad.BamRΔFib.pac. Once a heterologous fiber sequence is introduced in pBr/Ad.BamRΔFib.pac, the fiber modified right hand adenovirus clone may be introduced into a large cosmid clone as described for pWE/Ad.AflII-rITR in Example 1. Such a large cosmid clone allows generation of adenovirus by only one homologous recombination making the process extremely efficient.

<u>Amplification of fiber sequences from adenovirus serotypes</u>

To enable amplification of the DNAs encoding fiber protein derived from alternative serotypes degenerate oligonucleotides were synthesized. For this purpose, first known DNA sequences encoding fiber protein of alternative serotypes were aligned to identify conserved regions in both the tail-region as well as the knob-region of the fiber protein. From the alignment, which contained the nucleotide sequence of 19 different serotypes representing all 6 subgroups, (degenerate) oligonucleotides were synthesized (see Table 3). Also shown in table 3 is the combination of oligonucleotides used to amplify the DNA encoding fiber protein of a specific serotype. The amplification reaction (50 μl) contained 2 mM dNTPs, 25 pmol of each oligonucleotide, standard 1x PCR buffer, 1,5 mM $MgCl_2$, and 1 Unit Pwo heat stable polymerase (Boehringer) per reaction. The cycler program contained 20 cycles, each consisting of 30 sec. 94^0C, 60 sec. 60-64^0C, and 120 sec. at 72^0C. One-tenth of

the PCR product was run on an agarose gel which demonstrated that a DNA fragment was amplified. Of each different template, two independent PCR reactions were performed after which the independent PCR fragments obtained were sequenced to determine the nucleotide sequence. From 11 different serotypes, the nucleotide sequence could be compared to sequences present in GenBank. Of all other serotypes, the DNA encoding fiber protein was unknown till date and was therefore aligned with known sequences from other subgroup members to determine homology *i.e.*, sequence divergence. Of the 51 human serotypes known to date, all fiber sequences, except for serotypes 1, 6, and 26, have been amplified and sequenced. The protein sequences of the fiber from different adenovirus serotypes is given in FIG. 7.

Generation of fiber chimaeric adenoviral DNA constructs

All amplified fiber DNAs as well as the vector (pBr/Ad.BamRΔΔFib) were digested with NdeI and NsiI. The digested DNAs were subsequently run on a agarose gel after which the fragments were isolated from the gel and purified using the Geneclean kit (Bio101 Inc). The PCR fragments were then cloned into the NdeI and NsiI sites of pBr/AdBamRΔΔFib, thus generating pBr/AdBamRFibXX (where XX stands for the serotype number of which the fiber DNA was isolated). So far the fiber sequence of serotypes 5/ 7/ 8/ 9/ 10/ 11/ 12/ 13/ 14/ 16/ 17/ 19/ 21/ 24/ 27/ 28/ 29/ 30/ 32/ 33/ 34/ 35/ 36/ 37/ 38/ 40-S/ 40-L/ 41-S/ 42/45/ 47/ 49/ 51 have been cloned into pBr/AdBamRFibXX. From pBr/AdBamRFibXX (where XX is 5/ 8/ 9/ 10/ 11/ 13/ 16/ 17/ 24/ 27/ 30/ 32/ 33/ 34/ 35/ 38/ 40-S/ 40-L/ 45/ 47/ 49/ 51) an 6 kb AvrII fragment encompassing the fiber sequence was isolated via gelelectrophoresis and GeneClean. This AvrII fragment was subsequently cloned in plasmid

pBr/Ad.Bam-rITR.pac (see Example 1) which was digested to completion with AvrII and dephosphorylated as described previously, leading to the generation of the plasmid pBr/Ad.Bam-rITR.pac.fibXX. This plasmid was subsequently used to generate a cosmid clone with a modified fiber using the constructs pWE.pac, pBr/AflII-Bam and pBr/Ad.Bam-rITR.pac.fibXX. This cosmid cloning resulted in the formation of construct pWE/Ad.AflII-rITR/FibXX (where XX stands for the serotype number of which the fiber DNA was isolated).

Generation of pAd5/L420.HSA, pAd5/Clip and pAd5/Clipsal

pMLPI.TK was used to make a new vector in which nucleic acid molecules including specific promoter and gene sequences can be easily exchanged.

First, a PCR fragment was generated from pZipΔDMo+PyF101(N$^-$) template DNA (described in International Patent Application PCT/NL96/00195) with the following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' ~~(SEQ. ID. NO.)~~ (SEQ. ID. NO 58) and LTR-2: 5'-GCG GAT CCT TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3' ~~(SEQ. ID. NO.)~~ (SEQ. ID NO. 59). Pwo DNA polymerase (Boehringer Mannheim) was used according to manufacturers protocol with the following temperature cycles: once 5' at 95°°C; 3' at 55°°C; and 1' at 72°°C, and 30 cycles of 1' at 95°°C, 1' at 60°°C, 1' at 72°°C, followed by once 10' at 72°°C. The PCR product was then digested with BamHI and ligated into pMLP10 (Levrero et al., 1991; Gene 101, 195-202) digested with PvuII and BamHI, thereby generating vector pLTR10. This vector contains adenoviral sequences from bp 1 up to bp 454 followed by a promoter consisting of a part of the Mo-MuLV LTR having its wild-type enhancer sequences replaced by the enhancer from a

mutant polyoma virus (PyF101). The promoter fragment was designated L420. Sequencing confirmed correct amplification of the LTR fragment however the most 5' bases in the PCR fragment were missing so that the PvuII site was not restored. Next, the coding region of the murine HSA gene was inserted. pLTR10 was digested with BstBI followed by Klenow treatment and digestion with NcoI. The HSA gene was obtained by PCR amplification on pUC18-HSA (Kay et al., 1990; J. Immunol. 145, 1952-1959) using the following primers: HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA TGG TGG C-3' (SEQ. ID. NO. 60) and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT CTA CTA ACA GTA GAG ATG TAG AA-3' (SEQ. ID. NO. 61). The 269 bp amplified fragment was subcloned in a shuttle vector using the NcoI and BglII sites. Sequencing confirmed incorporation of the correct coding sequence of the HSA gene, but with an extra TAG insertion directly following the TAG stop codon. The coding region of the HSA gene, including the TAG duplication was then excised as a NcoI(sticky)-SalI(blunt) fragment and cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt) fragment from pLTR10, resulting in pLTR-HSA10.

Finally, pLTR-HSA10 was digested with EcoRI and BamHI after which the fragment containing the left ITR, packaging signal, L420 promoter and HSA gene was inserted into vector pMLPI.TK digested with the same enzymes and thereby replacing the promoter and gene sequences. This resulted in the new adapter plasmid pAd5/L420-HSA that contains convenient recognition sites for various restriction enzymes around the promoter and gene sequences. SnaBI and AvrII can be combined with HpaI, NheI, KpnI, HindIII to exchange promoter sequences, while the latter sites can be combined with the ClaI or BamHI sites 3' from HSA coding region to replace genes in this construct.

Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the promoter, gene and polyA sequences in pAd5/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a polyA signal. For this purpose, pAd5/L420-HSA was digested with AvrII and BglII followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pcDNA1/amp (Invitrogen) obtained by digestion with HhaI and AvrII followed by treatment with T4 DNA polymerase. This adapter plasmid was named pAd5/Clip. To enable removal of vector sequences from the adenoviral fragment pAd5/Clip was partially digested with EcoRI and the linear fragment was isolated. An oligo of the sequence 5' TTAAGTCGAC-3' (SEQ. ID. NO. 63) was annealed to itself resulting in a linker with a SalI site and EcoRI overhang. The linker was ligated to the partially digested pAd5/Clip vector and clones were selected that had the linker inserted in the EcoRI site 23 bp upstream of the left adenovirus ITR in pAd5/Clip resulting in pAd5/Clipsal.

Generation of pAd5ClipLacZ, pAd5Clip.Luc, pAd5Clip.TK and pAd5Clipsal.Luc

The adapter plasmid pAd5/Clip.LacZ was generated as follows: The E.coli LacZ gene was amplified from the plasmid pMLP.nlsLacZ (EPO Patent Application 95-202 213) by PCR with the primers 5'GGGGTGGCCAGGGTACCTCTAGGCTTTTGCAA (SEQ. ID. NO. 62) and 5'GGGGGGATCCATAAACAAGTTCAGAATCC (SEQ. ID. NO. 63). The PCR reaction was performed Ex Taq (Takara) according to the suppliers protocol at the following amplification program: 5 minutes 94°C, 1 cycle; 45 seconds 94°C and 30 seconds 60°C and 2 minutes 72°C, 5 cycles; 45 seconds 94°C and 30 seconds 65°C and 2 minutes 72°C, 25 cycles; 10 minutes 72; 45 seconds 94°C

and 30 seconds 60°C and 2 minutes 72°C, 5 cycles, I cycle. The PCR product was subsequently digested with Kpn1 and BamHI and the digested DNA fragment was ligated into KpnI/BamHI digested pcDNA3 (Invitrogen), giving rise to pcDNA3.nlsLacZ. Next, the plasmid pAd5/Clip was digested with SpeI. The large fragment containing part of the 5' part CMV promoter and the adenoviral sequences was isolated. The plasmid pcDNA3.nlsLacZ was digested with SpeI and the fragment containing the 3'part of the CMV promoter and the lacZ gene was isolated. Subsequently, the fragments were ligated, giving rise to pAd/Clip.LacZ. The reconstitution of the CMV promoter was confirmed by restriction digestion.

The adapter plasmid pAd5/Clip.Luc was generated as follows: The plasmid pCMV.Luc (EPO Patent Application 95-202 213) was digested with HindIII and BamHI. The DNA fragment containing the luciferase gene was isolated. The adapter plasmid pAd5/Clip was digested with HindIII and BamHI, and the large fragment was isolated. Next, the isolated DNA fragments were ligated, giving rise to pAd5/Clip.Luc. The adapter pClipsal.Luc was generated in the same way but using the adapter pClipsal digested with HIII and BamHI as vector fragment. Likewise, the TK containing HIII-BamHI fragment from pCMV.TK (EPO Patent Application 95-202 213) was inserted in pClipsal to generate pAd5/Clip.TK. The presence of the SalI site just upstream of the left ITR enables liberation of vector sequences from the adeno insert. Removal of these vector sequences enhances frequency of vector generation during homologous recombination in PER.C6.

Generation of recombinant adenovirus chimeric for fiber protein

To generate recombinant Ad 5 virus carrying the fiber of serotype 12, 16, 28, 40-L, 51, and 5, three constructs, pCLIP.Luc, pWE/AdAflII-Eco and pBr/AdBamrITR.pac/fibXX (XX = 12, 16, 28, 40-L, 51, and 5) were transfected into adenovirus

producer cells. To generate recombinant Ad 5 virus carrying the fiber of 5/ 7/ 8/ 9/ 10/ 11/ 12/ 13/ 14/ 16/ 17/ 19/ 21/ 24/ 27/ 28/ 29/ 30/ 32/ 33/ 34/ 35/ 36/ 37/ 38/ 40-S/ 40-L/ 41-S/ 42/45/ 47/ 49/ 51, two constructs pCLIP.Luc and pWE/Ad.AflII-rITR/FibXX were transfected into adenovirus producer cells. For transfection, 2 µg of pCLIP.Luc, and 4 µg of both pWE/AdAflII-Eco and pBr/AdBamrITR.pac/fibXX (or in case of cosmids: 4 µg of pCLIP.Luc plus 4 µg of pWE/Ad.AflII-rITR/FibXX) were diluted in serum free DMEM to 100 µl total volume. To this DNA suspension 100 µl 1x diluted lipofectamine (Gibco) was added. After 30 minutes at room temperature the DNA-lipofectamine complex solution was added to 2.5 ml of serum-free DMEM which was subsequently added to a T25 cm^2 tissue culture flask. This flask contained $2x10^6$ PER.C6 cells that were seeded 24-hours prior to transfection. Two hours later, the DNA-lipofectamine complex containing medium was diluted once by the addition of 2.4 ml DMEM supplemented with 20% fetal calf serum. Again 24 hours later the medium was replaced by fresh DMEM supplemented with 10% fetal calf serum. Cells were cultured for 6-8 days, subsequently harvested, and freeze/thawed 3 times. Cellular debris was removed by centrifugation for 5 minutes at 3000 rpm room temperature. Of the supernatant (12.5 ml) 3-5 ml was used to infect again infect PER.C6 cells (T80 cm^2 tissue culture flasks). This re-infection results in full CPE after 5-6 days after which the adenovirus is harvested as described above. With the generated virus batch two assays were routinely performed. 1) 20 µl virus supernatant, diluted 10-fold by the addition of 1980 µl DMEM was used to infect A549 cells that were seeded 24-hours prior to infection at a concentration of 10^5 cells per well of 6-well

plates. Forty-eight hours later protein lysates were prepared that were subsequently used to measure marker gene expression (luciferase activity). 2) 20 µl virus supernatant is used to determine the virus titer on human 911 cells. For this purpose, 911 cells are seeded at a concentration of $4x10^4$ cells per well in 96-well plates. Three to four hours after seeding, the medium was replaced by adenovirus supernatant (dilution range: 2 µl - 5×10^{-9} µl). The virus titers of the chimeric fiber Ad5 always exceeded 1×10^8 infectious units per ml.

Example 3: Production, purification, and titration of chimeric adenoviruses

Of the supernatant obtained from transfected PER.C6 cells, typically 10 ml was used to inoculate a 1 liter fermentor which contained 1 - 1.5 x 10^6 cells/ ml PER.C6 that were specifically adapted to grow in suspension. Three days after inoculation, the cells were harvested and pelleted by centrifuging for 10 min at 1750 rpm at room temperature. The chimeric adenoviruses present in the pelleted cells were subsequently extracted and purified using the following downstream processing protocol. The pellet was dissolved in 50 ml 10 mm $NaPO_4^-$ and frozen at -20°C. After thawing at 37°C, 5.6 ml deoxycholate (5% w/v) was added after which the solution was homogenated. The solution was subsequently incubated for 15 minutes at 37°C to crack the cells. After homogenizing the solution, 1875 µl (1M) $MgCl_2^-$ was added and 5 ml 100% glycerol. After the addition of 375 µl DNase (10 mg/ ml) the solution was incubated for 30 minutes at 37°C. Cell debris was removed by centrifugation at 1880xg for 30 minutes at room temperature without the brake on. The supernatant was subsequently purified from proteins by loading on 10 ml of FREON. Upon centrifugation

for 15 minutes at 2000 rpm without break at room temperature, three bands were visible of which the upper band represents the adenovirus. This band was isolated by pipetting after which it was loaded on a Tris/HCl (1M) buffered caesium chloride block gradient (range: 1.2 to 1.4 gr./ml). Upon centrifugation at 21000 rpm for 2.5 hours at 10°C the virus was purified from remaining protein and cell debris since the virus, in contrast to the other components, did not migrate into the 1.4 gr./ ml cesium chloride solution. The virus band was isolated after which a second purification using a Tris/ HCl (1M) buffered continues gradient of 1.33 gr./ml of cesium chloride is performed. After virus loading on top of this gradient the virus was centrifuged for 17 hours at 55000 rpm at 10°C. Subsequently the virus band was isolated and after the addition of 30 µl of sucrose (50 w/v) excess cesium chloride is removed by three rounds of dialysis, each round including of 1 hour. For dialysis the virus is transferred to dialysis slides (Slide-a-lizer, cut off 10000 kDa, Pierce, USA). The buffers used for dialysis are PBS which are supplemented with an increasing concentration of sucrose (round 1 to 3: 30 ml, 60 ml, and 150 ml sucrose (50% w/v)/ 1.5 liter PBS, all supplemented with 7.5 ml 2% (w/v) $CaMgCl_2$). After dialysis, the virus is removed from the slide-a-lizer after which it is aliquoted in portions of 25 and 100 µl upon which the virus is stored at -85°C.

To determine the number of virus particles per milliliter, 50 µl of the virus batch is run on an high performance liquid chromatograph columns (HPLC). The adenovirus is bound to the column (anion exchange) after which it is eluted using a NaCl gradient (range 300-600 mm). By determining the area under the virus peak, the number of virus particles can be calculated. To

determine the number of infectious units (IU) per ml present in a virus batch, titrations are performed on 911 cells. For this purpose, $4x10^4$ 911 cells are seeded per well of 96-well plates in rows B, D, and F in a total volume of 100 µl per well. Three hours after seeding the cells are attached to the plastic support after which the medium can be removed. To the cells a volume of 200 µl is added, in duplicate, containing different dilutions of virus (range: 10^2 times diluted to $2x10^9$). By screening for CPE the highest virus dilution which still renders CPE after 14 days is considered to contain at least one infectious unit. Using this observation, together with the calculated amount of virus volume present in these wells renders the number of infectious units per ml of a given virus batch. The production results *i.e.*,virus particles per ml and IU per ml or those chimeric adenoviruses that were produced so far, are shown in Table **4**.

Example 4: Re-directed infection of chimeric adenoviruses

To demonstrate re-directed infection *in vitro* of the adenoviruses chimeric for fiber protein, a panel of human cell lines of different origins was used. This panel includes, amongst others, human hepatic cells, primary fibroblasts, hemopoietic derived cell lines, primary smooth muscle cells, primary synoviocytes, and primary cells derived from the amniotic fluid such as amniocytes and chorion villi. These cell types were infected with a panel of chimeric adenoviruses which differ in the fiber protein. For this purpose, target cells are seeded at a concentration of 10^5 cells per well of 6-well plates in 2 ml Dulbecco's modified Eagle's medium (DMEM, Life Technologies, NL) supplemented with 10% Fetal calf serum. Twenty-four hours later the medium is replaced by fresh medium

containing the different chimeric adenoviruses at an increasing MOI of 0, 10, 50, 250, 1250, 2500, 5000 (MOI based on virus particles per cell). Approximately 2 hours after the addition of virus the medium containing the virus is discarded, cells are washed once with PBS, and subsequently 2 ml of fresh medium (not containing virus) is added to each well. Forty-eight hours later cells are harvested, washed and pelleted by centrifuging 5 minutes at 1500 rpm. Cells are subsequently lysed in 0,1 ml lysis buffer (1% Triton-X-100, 15% Glycerol, 2 mm EDTA, 2 mm DTT, and 25 mm $MgCl_2$ in Tris-phosphate buffer pH 7.8) after which the total protein concentration of the lysate is measured (Biorad, protein standard II). To determine marker gene expression (luciferase activity) 20 µl of the protein sample is mixed with 100 µl of a luciferase substrate (Luciferine, Promega, NL) and subsequently measured on a Lumat LB 9507 apparatus (EG & G Berthold, NL). The results of these infection experiments, given as the amount of luciferase activity (RLU) per µg protein, are shown in Table 5. These results clearly demonstrate that alteration of the fiber protein results in alteration of the Ad5 host range.

Example 5: Receptor usage of Fiber chimeric adenoviruses

To determine what cellular molecules are used by the fiber chimeric adenoviruses the expression of proteins known to be involved in Ad5 infection *i.e.*,Coxsackie adenovirus receptor (CAR), MHC class I, and integrins ($\alpha v\beta 3$, $\alpha v\beta 5$) was measured. For this purpose, $1x10^5$ target cells were transferred to tubes (4 tubes per cell type) designed for flow cytometry. Cells were washed once with PBS/ 0.5% BSA after which the cells were pelleted by centrifugation for 5 minutes at 1750 rpm at room temperature. Subsequently, 10 µl of a 100 times diluted $\alpha_v\beta 3$

antibody (Mab 1961, Brunswick Chemie, Amsterdam, NL), a 100 times diluted antibody $\alpha_v\beta5$ (antibody (Mab 1976, Brunswick Chemie), or 2000 times diluted CAR antibody (a kind gift of Dr. Bergelson, Harvard Medical School, Boston, USA (Hsu et al)) was added to the cell pellet after which the cells were incubated for 30 minutes at 4°C in a dark environment. After this incubation, cells were washed twice with PBS/0.5% BSA and again pelleted by centrifugation for 5 minutes at 1750 rpm room temperature. To label the cells, 10 µl of rat anti mouse IgG1 labeled with phycoerythrine (PE) was added to the cell pellet upon which the cells were again incubated for 30 minutes at 4°C in a dark environment. Finally the cells were washed twice with PBS/0.5% BSA and analyzed on a flow cytometer. The results of these experiments are shown in Table 6. Also, in Table 6 the infection efficiency of an adenovirus from subgroup A, B, C, D, and F is incorporated. These data clearly show that infection of a subgroup C adenovirus correlates with expression of CAR. The data also demonstrate that the chimeric adenoviruses carrying a fiber of an adenovirus of subgroup B, D, or F can infect cells that do not express measurable levels of the CAR protein thus being able to infect cells via different (CAR-independent) pathways.

Example 6: Radio-labeling of adenovirus particles

To enable tracking of infection of the wild type adenovirus serotypes, these viruses were labeled with radioactive I^{123}/I^{125} or with fluorescent probes prior to infection. Using fluorescent microscopy or by measuring radioactivity, the efficiency of infection of different serotypes into particular cell types is determined.

To demonstrate re-directed infection *in vivo* of adenovirus chimeric for fiber protein, $1x10^9$ infectious particles were

injected via the tail vein into CBA/ca mice (2 mice for each chimeric adenovirus). Detection of adenovirus infection into specific tissues is monitored on two different levels: 1)Binding of chimeric adenovirus is monitored by radioactive labeling the adenovirus (Eisenlohr et al., 1987; Matlin et al., 1981; Richman et al, 1998). One hour after in vivo systemic delivery via the tail vein mice are sacrificed after which preferred is investigated by measuring radioactivity in various organs c.q. tissues. 2) Successful infection is monitored by adenovirus gene expression of the marker gene *i.e.*, lacZ or luciferase activity. Four days after administration mice are sacrificed after which organs and tissues are isolated. Samples included liver, spleen, gastrointestinal tract, peripheral blood, bone marrow, aorta, muscle etc. Using this strategy, preferred binding of chimeric adenovirus towards tissues of interest can be investigated. Moreover, using this strategy, preferred infection of chimeric adenovirus into specific cells of particular organs can be determined.

80 µCi I^{123} (Cygne BV, NL) or I^{125} (Amersham) was activated by incubation for six minutes at RT in an Iodogen pre-coated tube (Pierce) in 100 µl iodination buffer (25 mm Tris, pH8, 0.4 M NaCl). The Radio-labeling reaction was started by transferring the activated Iodide to an Eppendorf tube containing $1,5.10^{10}$ adenovirus particles in 100 µl iodination buffer. The reaction was allowed to proceed for nine minutes at RT, after which incorporated label was separated from free label by gel filtration, using a Sephadex 25 column (P-10, Pharmacia). To this end, a P-10 column was pre-washed with 10 ml PBS buffer and subsequently loaded with the radio-labeling reaction, supplemented with two ml of iodination buffer. After discarding the first flow-through, the column was eluted with PBS buffer in 0.5 ml steps, and the different fractions were collected in separate tubes. Free label, which is slowed down by the column, was concentrated in fractions 10-16. Radio-labeled virus

particles accumulated predominantly in fractions 4, 5 and 6, corresponding to a total eluted volume of 2-3 ml. The radioactivity of these virus-containing fractions was measured and expressed as counts per minute (cpm), resulting in up to 5.10^6 cpm per 10^{10} virus particles.

Several control experiments were conducted to ensure the integrity of the virus particles after the various manipulations. For instance, one reaction was included in which the virus particles underwent identical treatment but with the omission of radioactive Iodide. Eluted virus particles were subsequently used to infect A549 cells. The amount of infected cells was established by the expression of a visual marker gene such as LacZ. In addition, small aliquots of those eluted fractions that represented radio-labeled adenovirus were used to infect A549 cells to test the expression of the transgene, which was taken as an indication for virus viability of the specific virus batch used.

The radio-labeled virus particles can subsequently be used for various *in vitro* and *in vivo* studies to determine the affinity for different cell types or for different organs. For *in vitro* studies, different cell lines such as for instance HUVEC (human umbilical vein endothelial cells) or SMC (smooth muscle cells) are seeded in 24-well plates in the appropriate culture medium, and infected with radio-labeled adenovirus particles at a MOI of 10, 100 and 1000. As a control, cells are incubated with a similar amount of free Iodide. Two hours after infection, cells are extensively washed with PBS buffer, and the remaining radioactivity measured. The amount of radioactivity that remains associated with the cells, corrected for the amount of radioactivity of the control cells incubated with free label, is a direct measure for the amount of virus that is attached to or has penetrated the cells.

For *in vivo* studies, the bio-distribution of adenoviruses that differ only in the origin of their fiber proteins was compared. To this end, rats were placed under general anesthetic and 0.1-2 MBq of radio-labeled adenovirus particles was intravenously (iv) administered into the tail vein. As a control, one rat received a comparable dose of free Iodide only. The animals were subsequently placed onto a gamma scanner and scanned for 10 minutes, to localize the source of the gamma radiation and thus to determine the in vivo bio-distribution of systemically introduced adenovirus. After one hour, animals were sacrificed and the major organs removed for weighing and for accurate quantification of radioactivity using a scintillation counter. The distribution of radioactivity in various organs after iv is expressed as cpm per gram tissue, and is shown in FIG. 8.

Example 7: Infection of human primary cells from amniotic fluid.

In Table 5 (Example 4) infection results are shown on both amniotic cells and chorion villi. These cell types are isolated from the amniotic fluid and cultured ex vivo under standard conditions (Roest et al, 1996). Such cells are ideal targets to use for prenatal diagnosis. For instance, in some cases (approximately 50-100 yearly) prenatal diagnosis of muscular dystrophin is impossible using standard techniques such as reverse- transcribed PCR or DNA PCR because the mutations in the dystrophin gene are unknown and the level of dystrophin produced in non-differentiated chorionvilli or amnionvilli cells is very low. In these cases isolation and fast differentiation of predominantly chorionvilli cells is performed. These chorionvilli are subsequently infected with a

retrovirus (Roest et al, 1996) or an adenovirus carrying the MyoD cDNA (Roest et al, 1999) which, upon transduction, triggers the chorionvilli to differentiate into striated muscle cells within one week. After complete differentiation these cells can then be used for Western analysis, or immunohistochemistry to determine whether the dystrophin protein is expressed. To date, the infection efficiency of chorionvilli cells has been disappointing with only 2-5% of cells transduced with a retrovirus (Roest et al, 1996). Using a serotype 5 adenovirus to deliver the MyoD cDNA to chorionvilli approximately 10%-20% (Roest et al, 1999) of the cells can be transduced but only when using high multiplicity of infection ("MOI") which results in undesired toxicity and thus cell death. The results in Table 5 clearly demonstrate that the Ad5 is not an ideal candidate for transducing chorionvilli cells since only marginal luciferase activity is measured (75 RLU/ µg protein) at the highest MOI tested (MOI = 5000 virus particles per cell). These results are confirmed using flow cytometry for the presence of the Coxsackie adenovirus receptor (CAR) and integrins which demonstrates that the receptors for Ad5 are only marginally present on chorionvilli (Table 6). Surprisingly, the Ad5 based vector containing a fiber of either subgroup B (fiber 16 and/or 51) or subgroup F (fiber 40-L) both transduce the chorionvilli with high efficiency. The vector which does best, based on luciferase activity is the adenovirus 5 with fiber 40-L which results in 1,688,028 relative light units per µg of protein, >20,000 fold increased transgene expression as compared to Ad5. This vector can thus be used to transduce cells present in the amniotic fluid to allow fast differentiation for purposes described above, for inhibiting gene expression during prenatal

development, or to transfer and express nucleic acid of interest to the amniotic fluid.

Example 8: Generation of Ad5 based viruses with chimeric hexon protein.

The method described *infra* to generate recombinant adenoviruses by co-transfection of two, or more separate cloned adenovirus sequences. These cloned adenoviral sequences were subsequently used to remove specific Ad5 sequences in order to generate template clones which allow for the easy introduction of DNA sequences derived from other adenovirus serotypes. As an example of these template clones, the construction of plasmids enabling swapping of DNA encoding for hexon protein is given.

<u>Generation of adenovirus template clones lacking DNA encoding for hexon</u>

Hexon coding sequences of Ad5 are located between nucleotides 18841 and 21697. To facilitate easy exchange of hexon coding sequences from alternative adenovirus serotypes into the Ad5 backbone, first a shuttle vector was generated. This subclone, coded pBr/Ad.Eco-PmeI, was generated by first digesting plasmid pBr322 with EcoRI and EcoRV and inserting the 14 kb PmeI-EcoRI fragment from pWE/Ad.AflII-Eco. In this shuttle vector a deletion was made of a 1430 bp SanDI fragment by digestion with SanDI and religation to give pBr/Ad.Eco-PmeI ΔSanDI. The removed fragment contains unique SpeI and MunI sites. From pBr/Ad.Eco-PmeIΔSanDI the Ad5 DNA encoding hexon was deleted. Hereto, the hexon flanking sequences were PCR amplified and linked together thereby generating unique restriction sites replacing the hexon coding region. For these

PCR reactions four different oligonucleotides were required: Δhex1-Δhex4. Δhex1: 5'- CCT GGT GCT GCC AAC AGC-3' ~~(SEQ. ID. NO.~~ (SEQ. ID. NO. 65)

Δhex2: 5'- CCG GAT CCA CTA GTG GAA AGC GGG CGC GCG-3' ~~(SEQ. ID. NO.~~ (SEQ ID NO. 66)

Δhex3: 5'- CCG GAT CCA ATT GAG AAG CAA GCA ACA TCA ACA AC-3' ~~(SEQ. ID. NO.~~ (SEQ. ID. NO. 67)

Δhex4: 5'- GAG AAG GGC ATG GAG GCT G-3' ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 68) (See FIG. 9).

The amplified DNA product of ± 1100 bp obtained with oligonucleotides Δhex1 and Δhex2 was digested with BamHI and FseI. The amplified DNA product of ± 1600 bp obtained with oligonucleotides Δhex3 and Δhex4 was digested with BamHI and SbfI. These digested PCR fragments were subsequently purified from agarose gel and in a tri-part ligation reaction using T4 ligase enzyme linked to pBr/Ad.Eco-PmeI ΔSanDI digested with FseI and SbfI. The resulting construct was coded pBr/Ad.Eco-PmeΔHexon. This construct was sequenced in part to confirm the correct nucleotide sequence and the presence of unique restriction sites MunI and SpeI.

Amplification of hexon sequences from adenovirus serotypes

To enable amplification of the DNAs encoding hexon protein derived from alternative serotypes degenerate oligonucleotides were synthesized. For this purpose, first known DNA sequences encoding for hexon protein of alternative serotypes were aligned to identify conserved regions in both the N-terminus as well as the C-terminus of the Hexon protein. From the alignment, which contained the nucleotide sequence of 9 different serotypes representing 5 of the 6 known subgroups,

(degenerate) oligonucleotides were synthesized. These oligonucleotides were coded HEX-up (5'- GG ACGTGT AAG ATG GCY ACC CCH TCG ATG MTG- 3') (SEQ. ID. NO. 69) and HEX-down (5'- CCA TCG ATG GTT ATG TKG TKG CGT TRC CGG C -3') (SEQ. ID. NO. 70). The amplification reaction (50 µl) contained 2 mM dNTPs, 25 pmol of each oligonucleotide, standard 1x PCR buffer, 1,5 mM $MgCl_2$, and 1 Unit Pwo heat stable polymerase (Boehringer) per reaction. The cycler program contained 20 cycles, each consisting of 30 sec. 94°C, 60 sec. 60-64°C, and 120 sec. At 72°C. One-tenth of the PCR product was run on an agarose gel which demonstrated that a DNA fragment was amplified. Of each different template, two independent PCR reactions were performed after which the independent PCR fragments obtained were sequenced to determine the nucleotide sequence. From 9 different serotypes, the nucleotide sequence could be compared to sequences present in GenBank. Of all other serotypes, the nucleotide sequence encoding the Hexon protein is unknown. So far, of each serotype, except for serotypes 1, 8, 13, and 18, the hexon sequence has been PCR amplified. The protein sequence of the hexon of serotypes 34, 35, 36, and 41 is given in FIG. 10.

Generation of Hexon chimaeric adenoviral DNA constructs

All amplified hexon DNAs as well as the vector (pBr/Ad.Eco-PmeΔHexon) were digested with MunI and SpeI. The digested DNAs was subsequently run on a agarose gel after which the fragments were isolated from the gel and purified using the Geneclean kit (Bio101 Inc). The PCR fragments were then cloned into the MunI and SpeI sites of pBr/Ad.Eco-PmeΔHexon, thus generating pBr/Ad.Eco-PmeΔHexXX (where XX stands for the serotype number of which the fiber DNA was isolated). So far

the hexon sequence of serotypes 2, 3, 4, 5, 7, 9, 10, 11, 14, 15, 16, 19, 20, 22, 23, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 40, 41, 42, 43, 46, 47, 48, 49, 50, 51 have been cloned into pBr/Ad.Eco-PmeΔHexXX. From pBr/Ad.Eco-PmeΔHexXX (where XX is 20, 25, 26, 28, 30, 34, 35) a 9.6 kb AscI fragment encompassing the hexon sequence was isolated via gel electrophoresis and an agarase protocol (Boehringer Mannheim, NL). This AscI fragment was subsequently cloned in cosmid pWE/Ad.AflII-rITRsp (see, Example 1) which was digested to completion with AscI and deposphorylated as described previously. This cosmid cloning resulted in the formation of construct pWE/Ad.AflII-rITR/HexXX (where XX stands for the serotype number of which the hexon DNA was isolated)

Generation of recombinant adenovirus chimeric for hexon protein

To generate recombinant Ad 5 virus carrying the hexon of alternative serotypes two constructs, pCLIP.Luc, pWE/Ad.AflII-rITR/HexXX were transfected into adenovirus producer cells. For transfection, 4 μg of pCLIP.Luc, and 4 μg of pWE/Ad.AflII-rITR/HexXX were diluted in serum free DMEM to 100 μl total volume. To this DNA suspension 100 μl 2/3 x diluted lipofectamine (Gibco) was added. After 30 minutes at room temperature the DNA-lipofectamine complex solution was added to 2.5 ml of serum-free DMEM which was subsequently added to a T25 cm^2 tissue culture flask (cells washed with 5 ml serum free medium prior to addition of DNA-lipofectamine complex). This flask contained 3 x 10^6 PER.C6 cells that were seeded 24-hours prior to transfection. Two hours later, the DNA-lipofectamine complex containing medium was diluted once by the addition of 2.5 ml DMEM supplemented with 20% fetal calf serum. Again 24 hours later the medium was replaced by fresh DMEM supplemented with 10% fetal calf serum. Cells were cultured for 6-8 days,

subsequently harvested, and freeze/thawed. Cellular debris was removed by centrifugation for 5 minutes at 3000 rpm room temperature. Of the supernatant (12.5 ml) 3-5 ml was used to again infect PER.C6 cells (T80 cm^2 tissue culture flasks).

Re-directed neutralization towards hexon chimeric adenovirus

To demonstrate an altered immune response towards chimeric adenoviruses, we first tested 75 sera derived from human patients (25 cancer patients, 50 rheumatoid arthritis patients) for toxicity on human 911 cells. For this purpose, 911 cells were seeded at a concentration of $3x10^4$ cells per well in 96-well microtiter plates. Twenty-four hours later the medium of all wells, except for wells A1-H1, A5-H5, and A9-H9, was replaced by 50 µl DMEM supplemented with 5% fetal calf serum. To wells A1, A2, B1, and B2, 50 µl patient serum 1 was added. Likewise, To wells C1, C2, D1, and D2, 50 µl of patient serum 2 was added etc. Subsequently, 50 µl of wells A2-H2 were transferred to A3-H3 after which 50 µl of wells A3-H3 was transferred to A4-H4. Thus this test schedule resulted in a serum dilution of 0x, 2x, 4x, and 8x for each patient serum. Identical treatment of wells A5-H5 through A8-H8, and A9-H9 through A12-H12 results in 12 sera tested per 96-well microtiter plate. From 75 human patient sera tested in total, 25 sera with no apparent toxicity on human 911 cells were subsequently tested for the presence of antibodies capable of neutralizing chimeric adenovirus infection. For this purpose, 96-well microtiter plates were filled with 50 µl DMEM supplemented with 5% fetal calf serum except for wells A1-H1. To wells A1, A2, B1, and B2, 50 µl patient serum 1 was added. Likewise, to wells C1, C2, D1, and D2, 50 µl patient serum 2

was added etc. Subsequently, 50 µl of wells A2-H2 were transferred to wells A3-A4 after which 50 µl of A3-H3 was transferred to A4-H4 etc. until A12-H12 (dilution range: 0 - 1/2048). From wells A12-H12, 50 µl was discarded. Next, 50 µl of virus was added after which the microtiter plates were incubated for 1 hour at 37°C. Upon the addition of 50 µl 911 cell-suspension (3 x 10^4 cells/ well) plates were incubated for 7-9 days after which neutralizing capacity was scored by the absence, presence, or severity of CPE. As controls during these experiments absence of serum, absence of virus, and absence of serum and virus were taken. Based on these experiments several chimeric viruses are identified towards which little neutralizing antibodies are generated by humans. Similar experiments as described above are performed with wildtype adenovirus serotypes from both human as well as animals to screen for serotypes which are less prone to neutralization due to the host defense system. These experiments although similar are developed in such a way that it allows high throughput screening of many samples at once. This assay is described below.

<u>A high throughput assay for the detection of neutralizing activity in human serum</u>

To enable screening of a large amount of human sera for the presence of neutralizing antibodies against all adenovirus serotypes, an automated 96-wells assay was developed.

Human sera

A panel of 100 individuals was selected. Volunteers (50% male, 50% female) were healthy individuals between 20 and 60 years old with no restriction for race. All volunteers signed

an informed consent form. People professionally involved in adenovirus research were excluded.

About 60 ml blood was drawn in dry tubes. Within 2 hours of sampling, the blood was centrifuged at 2500 rpm for 10 min. Approximately 30 ml serum were transferred to polypropylene tubes and stored frozen at -20°C until further use.

Serum was thawed and heat-inactivated at 56°C for 10 minutes and then aliquoted to prevent repeated cycles of freeze/thawing. Part was used to make five steps of twofold dilutions in medium (DMEM, Gibco BRL) in a quantity enough to fill out approximately 70 96-well plates. Aliquots of undiluted and diluted sera were pipetted in deep well plates (96-well format) and using a programmed platemate dispensed in 100 µl aliquots into 96-well plates. This way the plates were loaded with eight different sera in duplo (100 µl/well) according to the scheme below:

S1/2	S1/4	S1/8	S1/16	S1/32	S5/2	S5/4	S5/8	S5/16	S5/32	-	-
S1/2	S1/4	S1/8	S1/16	S1/32	S5/2	S5/4	S5/8	S5/16	S5/32	-	-
S2/2	S2/4	S2/8	S2/16	S2/32	S6/2	S6/4	S6/8	S6/16	S6/32	-	-
S2/2	S2/4	S2/8	S2/16	S2/32	S6/2	S6/4	S6/8	S6/16	S6/32	-	-
S3/2	S3/4	S3/8	S3/16	S3/32	S7/2	S7/4	S7/8	S7/16	S7/32	-	-
S3/2	S3/4	S3/8	S3/16	S3/32	S7/2	S7/4	S7/8	S7/16	S7/32	-	-
S4/2	S4/4	S3/8	S3/16	S3/32	S8/2	S8/4	S8/8	S8/16	S8/32	-	-
S4/2	S4/4	S3/8	S3/16	S3/32	S8/2	S8/4	S8/8	S8/16	S8/32	-	-

Where S1/2 to S8/2 in columns 1 and 6 represent 1x diluted sera and Sx/4, Sx/8, Sx/16 and Sx/32 the twofold serial dilutions. The last plates also contained four wells filled with 100 µl fetal calf serum as a negative control.

Plates were kept at -20°C until further use.

Preparation of human adenovirus stocks

Prototypes of all known human adenoviruses were inoculated on T25 flasks seeded with PER.C6 cells (Fallaux *et al.*, 1998) and harvested upon full CPE. After freeze/thawing 1-2 ml of the crude lysates were used to inoculate a T80 flask with PER.C6 cells and virus was harvested at full CPE. The time frame between inoculation and occurrence of CPE as well as the amount of virus needed to re-infect a new culture, differed between serotypes. Adenovirus stocks were prepared by freeze/thawing and used to inoculate 3-4 T175 cm^2 three-layer flasks with PER.C6 cells. Upon occurrence of CPE, cells were harvested by tapping the flask, pelleted and virus was isolated and purified by a two step CsCl gradient as follows. Cell pellets were dissolved in 50 ml 10 mm $NaPO_4$ buffer (pH 7.2) and frozen at -20°C. After thawing at 37°C, 5.6 ml sodium deoxycholate (5% w/v) was added. The solution was mixed gently and incubated for 5-15 minutes at 37°C to completely lyse the cells. After homogenizing the solution, 1875 μl 1M $MgCl_2$ was added. After the addition of 375 μl DNase (10 mg/ml) the solution was incubated for 30 minutes at 37°C. Cell debris was removed by centrifugation at 1880xg for 30 minutes at RT without brake. The supernatant was subsequently purified from proteins by extraction with FREON (3x). The cleared supernatant was loaded on a 1M Tris/HCl buffered cesium chloride block gradient (range: 1.2/1.4 gr/ml) and centrifuged at 21000 rpm for 2.5 hours at 10°C. The virus band is isolated after which a second purification using a 1M Tris/HCl buffered continues gradient of 1.33 gr/ml of cesium chloride was performed. The virus was then centrifuged for 17 hours at 55000 rpm at 10°C. The virus band is isolated and sucrose (50 % w/v) is added to a final concentration of 1%. Excess CsCl is removed by dialysis (three times 1 hr at RT) in dialysis slides (Slide-a-lizer, cut

off 10000 kDa, Pierce, USA) against 1.5 ltr PBS supplemented with $CaCl_2$ (0.9 mm), $MgCl_2$ (0.5mM) and an increasing concentration of sucrose (1, 2, 5%). After dialysis, the virus is removed from the slide-a-lizer after which it is aliquoted in portions of 25 and 100 µl upon which the virus is stored at -85°C.

To determine the number of virus particles per milliliter, 50 µl of the virus batch is run on a high-pressure liquid chromatograph (HPLC) as described by Shabram et al (1997). Viruses were eluted using an NaCl gradient ranging from 0 to 600 mm. As depicted in table I, the NaCl concentration by which the viruses were eluted differed significantly among serotypes.

Most human adenoviruses replicated well on PER.C6 cells with a few exceptions. Adenovirus types 8 and 40 were grown on 911-E4 cells (He *et al.*, 1998). Purified stocks contained between $5x10^{10}$ and $5x10^{12}$ virus particles/ml (VP/ml)

Titration of purified human adenovirus stocks

Adenoviruses were titrated on PER.C6 cells to determine the amount of virus necessary to obtain full CPE in five days, the length of the neutralization assay. Hereto, 100 µl medium was dispensed into each well of 96-well plates. 25 µl of adenovirus stocks pre-diluted 10^4, 10^5, 10^6 or 10^7 times were added to column 2 of a 96-well plate and mixed by pipetting up and down 10 times. Then 25 µl was brought from column 2 to column 3 and again mixed. This was repeated until column 11 after which 25 µl from column 11 was discarded. This way serial dilutions in steps of 5 were obtained starting off from a pre-diluted stock. Then $3x10^4$ PER.C6 cells were added in a 100 µl volume and the plates were incubated at 37 °C, 5% CO_2 for five or six days. CPE was monitored microscopically. The method of

Reed and Muensch was used to calculate the cell culture inhibiting dose 50% (CCID50).

In parallel, identical plates were set up that were analyzed using the MTT assay (Promega). In this assay living cells are quantified by colorimetric staining. Hereto, 20 µl MTT (7.5 mgr/ml in PBS) was added to the wells and incubated at 37 ºC, 5% CO_2 for two hours. The supernatant was removed and 100 µl of a 20:1 isopropanol/triton-X100 solution was added to the wells. The plates were put on a 96-wells shaker for 3-5 minutes to solubilize precipitated staining. Absorbance was measured at 540 nm and at 690 nm (background). By this assay wells with proceeding CPE or full CPE can be distinguished.

Neutralization assay

96-well plates with diluted human serum samples were thawed at 37 ºC, 5% CO_2. Adenovirus stocks diluted to 200 CCID50 per 50 µl were prepared and 50 µl aliquots were added to columns 1-11 of the plates with serum. Plates were incubated for 1 hour at 37ºC, 5% CO_2. Then 50 µl PER.C6 cells at $6x10^5$/ml were dispensed in all wells and incubated for 1 day at 37 ºC, 5% CO_2. Supernatant was removed using fresh pipette tips for each row and 200 µl fresh medium was added to all wells to avoid toxic effects of the serum. Plates were incubated for another 4 days at 37 ºC, 5% CO_2. In addition, parallel control plates were set up in duplo with diluted positive control sera generated in rabbits and specific for each serotype to be tested in rows A and B and with negative control serum (FCS) in rows C and D. Also, in each of the rows E-H a titration was performed as described above with steps of five times dilutions starting with 200 CCID50 of each virus to be tested. On day 5 one of the control plates was analyzed microscopically and with

the MTT assay. The experimental titer was calculated from the control titration plate observed microscopically. If CPE was found to be complete, *i.e.*, the first dilution in the control titration experiment analyzed by MTT shows clear cell death, all assay plates were processed. If not, the assay was allowed to proceed for one or more days until full CPE was apparent after which all plates were processed. In most cases the assay was terminated at day 5. A serum sample is regarded to be non-neutralizing when at the highest serum concentration a maximum protection is seen of 40% compared to the controls without serum.

Example 9: Generation of Ad5 based viruses with chimeric penton proteins

The method described *infra* to generate recombinant adenoviruses by co-transfection of two, or more separate cloned adenovirus sequences. These cloned adenoviral sequences were subsequently used to remove specific Ad5 sequences in order to generate template clones which allow for the easy introduction of DNA sequences derived from other adenovirus serotypes. As an example of these template clones, the construction of plasmids enabling swapping of DNA encoding for penton protein is given.

<u>Generation of adenovirus template clones lacking DNA encoding for penton</u>

First a shuttle vector for penton sequences was made by insertion of the 7.2 kb NheI-EcoRV fragment from construct pWE/Ad.AflII-EcoRI (described in example 1) into pBr322 digested with the same enzymes. The resulting vector was named pBr/XN. From this plasmid Ad5 penton sequences were deleted and replaced by unique restriction sites that are then used to introduce new penton sequences from other serotypes. Hereto,

the left flanking sequences of penton in pBr/XN were PCR amplified using the following primers:

DP5-F: 5'- CTG TTG CTG CTG CTA ATA GC-3' ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 71) and

DP5-R: 5'- CGC GGA TCC **TGT ACA** ACT AAG GGG AAT ACA AG-3' ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 72)

DP5-R has an BamHI site (underlined) for ligation to the right flanking sequence and also introduces a unique BsrGI site (bold face) at the 5'-end of the former Ad5 penton region.

The right flanking sequence was amplified using:

DP3-F: 5'-CGC GGA TCC **CTT AAG** GCA AGC ATG TCC ATC CTT-3' ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 73) and

DP3-3R: 5'- AAA ACA CGT TTT ACG CGT CGA CCT TTC-3' ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 74)

DP3-F has an BamHI site (underlined) for ligation to the left flanking sequence and also introduces a unique AflII site (bold face) at the 3'-end of the former Ad5 penton region.

The two resulting PCR fragments were digested with BamHI and ligated together. Then this ligation mixture was digested with AvrII and BglII. pBr/XN was also digested with AvrII and BglII and the vector fragment was ligated to the digested ligated PCR fragments. The resulting clone was named pBr/Ad.Δpenton. Penton coding sequences from serotypes other than Ad5 were PCR amplified such that the 5' and 3' ends contained the BsrGI and AflII sites respectively. Introduction of these heterologous penton sequences in pBr/Ad.Δpenton generates constructs named pBr/Ad.pentonXX where XX represents the number of the serotype corresponding to the serotype used to amplify the inserted penton sequences. Subsequently, the new penton sequences were introduced in the pWE/Ad.AflII-rITR construct by exchanging the common FseI fragment. Importantly, in stead of pWE/Ad.AflII-rITR it is also possible to insert the

FseI fragment from pBr/Ad.pentonXX into a pWE/Ad.AflII-rITR/HexXX or an pWE/Ad.AflII-rITR/FibXX vector having a modified hexon and/or fiber sequence respectively. In this way the plasmid-based system to generate adenoviruses enables flexible design of any adenovirus with any desired characteristic concerning efficiency and specificity of infection of the target cell as well as immunogenicity.

Amplification of penton sequences from adenovirus serotypes

To enable amplification of the DNAs encoding penton protein derived from alternative serotypes oligonucleotides were synthesized. Of each adenovirus subgroup the penton sequence of only one member is known to date. Therefore, oligonucleotides were designed based on the known sequences Thus, for amplification of penton sequences from subgroup C oligonucleotides P5-for (5'-gctcgatgtacaatgcggcgcgcggcgatgtat-3') ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 75) and P5-rev (5'-gctcgacttaagtcaaaaagtgcggctcgatag-3') ~~(SEQ. ID. NO. __)~~ (SEQ ID NO. 76) were used .For the amplification of penton sequences from subgroup B oligonucleotides P3-for (5'gctcgatgtacaatgaggagacgagccg tgcta-3') ~~(SEQ. ID. NO. __)~~ (SEQ. ID NO. 77) and P3-rev (5'-gctcgacttaagttagaaagtgcggcttgaaag-3') ~~(SEQ. ID. NO. __)~~ (SEQ. ID NO. 78) were used. For the amplification of penton sequences from subgroup D oligonucleotides P17-for (5'gctcgatgtacaatgaggcgt gcggtggtgtcttc-3') ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 79) and P17-rev (5'-gctcgacttaagttagaaggtgcg actggaaagc-3') ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 81) were used. For the amplification of penton sequences from subgroup F oligonucleotides PF-for (5'-gctcgatgtacaatgagacgtgcggtgggagtg-3') ~~(SEQ. ID. NO. __)~~ (SEQ. ID. NO. 82) and PF-rev (5'-gctcga cttaagttaaaacgtgcggctagacag-3') ~~(SEQ. ID. NO. __)~~ (SEQ ID NO. 83) were used. All above described forward oligonucleotides contain a BsrGI restriction site at their 5'-end and all reverse

oligonucleotides contain an AflII restriction site at the 5'-end.

The amplification reaction (50 µl) contained 2 mm dNTPs, 25 pmol of each oligonucleotide, standard 1x PCR buffer, 1,5 mm $MgCl_2$, and 1 Unit Pwo heat stable polymerase (Boehringer) per reaction. The cycler program contained 20 cycles, each consisting of 30 sec. 94°C, 60 sec. 60-64°C, and 120 sec. At 72°C. One-tenth of the PCR product was run on an agarose gel which demonstrated that a DNA fragment was amplified. Of each different template, two independent PCR reactions were performed after which the independent PCR fragments obtained are sequenced to determine the nucleotide sequence. Of the 51 human serotypes 20 penton sequences have been amplified.

Generation of penton chimeric adenoviral DNA constructs

All amplified penton DNAs as well as the vector (pBr/Ad.Δpenton) were digested with BsrGI and AflII. The digested DNAs was subsequently run on a agarose gel after which the fragments were isolated from the gel and purified using the Geneclean kit (Bio101 Inc). The PCR fragments were then cloned into the BsrGI and AflII sites of pBr/Ad.Δpenton, thus generating pBr/Ad.pentonXX (where XX stands for the serotype number of which the penton DNA was isolated). So far the penton sequence of serotypes 2, 3, 5, 6, 7, 11, 21, 26, 35, 39, 40, 41, 42, 47, 48, 49 and 51 have been cloned into pBr/Ad.pentonXX . From pBr/Ad.pentonXX an 5.1 kb FseI fragment encompassing the penton sequence was isolated via gel electrophoresis and Geneclean. This FseI fragment was subsequently cloned in cosmid pWE/Ad.AflII-rITR (see, Example 1) which was digested to completion with FseI and deposphorylated as described previously. This cosmid cloning resulted in the formation of

construct pWE/Ad.AflII-rITR/PentonXX (where XX stands for the serotype number of which the penton DNA was isolated).

Generation of recombinant adenovirus chimaeric for penton protein

To generate recombinant Ad 5 virus carrying the Penton of alternative serotypes two constructs, pCLIP.Luc and pWE/Ad.AflII-rITR/PenXX were transfected into adenovirus producer cells.

For transfection, 4 µg of pCLIP.Luc and 4 µg of pWE/Ad.AflII-rITR/PentonXX) were diluted in serum free DMEM to 100 µl total volume. To this DNA suspension 100 µl 1x diluted lipofectamine (Gibco) was added. After 30 minutes at room temperature the DNA-lipofectamine complex solution was added to 2.5 ml of serum-free DMEM which was subsequently added to a T25 cm^2 tissue culture flask. This flask contained $2x10^6$ PER.C6 cells that were seeded 24-hours prior to transfection. Two hours later, the DNA-lipofectamine complex containing medium was diluted once by the addition of 2.5 ml DMEM supplemented with 20% fetal calf serum. Again 24 hours later the medium was replaced by fresh DMEM supplemented with 10% fetal calf serum. Cells were cultured for 6-8 days, subsequently harvested, and freeze/thawed 3 times. Cellular debris was removed by centrifugation for 5 minutes at 3000 rpm room temperature. Of the supernatant (12.5 ml) 3-5 ml was used to infect again infect PER.C6 cells (T80 cm^2 tissue culture flasks). This re-infection results in full CPE after 5-6 days after which the adenovirus is harvested as described above.

The described Examples 1-9 encompass the construction of recombinant adenoviral vectors, chimaeric for either fiber protein or hexon protein which results in an altered infection

host range or altered immune response towards adenoviral vectors. These chimaeric adenoviral vectors are generated for the purpose of gene transfer and recombinant DNA vaccines. It must be stressed that in a manner analogous as described under Examples 1-9, chimeric adenoviral vectors are constructed for penton and can be constructed for all other adenovirus proteins including but not limited to DNA encoding for small proteins required for adenovirus assembly and sequences required for adenovirus replication. Moreover, it must be emphasized that with this technology double, triple, quadruple, etc. chimeric adenoviral vectors can be constructed with the aim to combine parts of existing adenovirus serotypes to generate adenoviral vectors with preferred characteristics for any given target cell or target disease.

Legends to Tables

Table 1: Summary of the classification of known human adenovirus serotypes based upon the principle of hemagglutination.

Table 2: Association of human adenovirus serotypes with human disease.

Table 3: Oligonucleotides and degenerate oligonucleotides used for the amplification of DNA encoding for fiber protein derived from alternative human adenovirus serotypes. Bold letters in oligonucleotides A-E represent an NdeI restriction site. Bold letters in oligonucleotides 1-6 and 8 represent an NsiI restriction site. Bold letters in oligonucleotide 7 represent a PacI restriction site.

Table 4: Production results of fiber chimeric adenoviruses. The number of virus particles per ml were determined using HPLC. The number of infectious units (IU) per milliliter were determined through titration on human 911 cells. For infection experiments, the number of virus particles per milliliter is taken from all chimeric adenoviruses since IU/ ml reflects a receptor mediated process.

Table 5: Transduction results of human cell lines and primary cells. A549: Human lung carcinoma cell line (ATCC, CCL-1185). K562: Human erythroid leukemia (ATCC, CCL-243). SupT1: Human Lymphoblast hybrid B and T (ATCC, CRL-1991). GM09503: Human primary fibroblasts. HEPG2: Human liver carcinoma (ATCC, HB8065). CEM: human lymphoblast cells (ATCC, CRL-1992). HeLa: Human cervix carcinoma (ATCC, CCL-2). Primary amniocytes and chorionvilli cells were obtained from department of antropogenetics, Leiden, NL. Primary Smooth muscle cells and synoviocytes were obtained from TNO-PG, Leiden, The Netherlands. Shown are the luciferase activity (in relative light units (RLU) per µg protein) measurements of cells infected at MOI 5000 virus particles per cell.

Table 6: Expression of integrins $\alpha_v\beta3$ and $\alpha_v\beta5$, the Coxsackie adenovirus receptor (CAR), and MHC class I on the membranes of target cells. In addition to the cells described in table 5: HUVEC: human umbilical vein endothelial cells were obtained from TNO-PG, Leiden, The Netherlands. Shown is the percentage of cells expressing either molecule on their membrane. The Ad5 based vector carrying a fiber of one representative of each subgroup and the efficiency of infection is shown on the right of the table. ND: not determined. 0% means undetectable

expression of the molecule on the membrane of the cell using flow cytometry. 100% means high expression of the molecule on the cell membrane.

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Table 1

Subgroup	serotypes	hemagglutination rhesus	hemagglutination rat
A	12, 18, 31	-	+/-
B	3, 7, 11, 14, 16, 21, 34, 35, 51	+	-
C	1, 2, 5, 6	-	+/-
D	8-10, 13, 15, 17, 19, 20, 22-30 ,32, 33, 36-39, 42-47, 49, 50	+/-	+
E	4	-	+/-
F	40, 41	-	+/-

Table 2

Syndrom	Subgenus	Serotype
Respiratory illness	A	31
	B	3, 7, 11, 14, 21, 34, 35, 51
	C	1, 2, 5, 6
	D	39, 42-48
	E	4
Keratoconjunctivitis (eye)	B	11
	D	8, 19, 37, 50
Hemorrhagic cystitis (Kidney)	B	7, 11, 14, 16, 21, 34, 35
And urogenital tract infections	C	5
	D	39, 42-48
Sexual transmission	C	2
	D	19, 37
Gastroenteritis	A	31
	B	3
	C	1, 2, 5
	D	28
	F	40, 41
CNS disease	A	12, 31
	B	3, 7
	C	2, 5, 6
	D	32, 49
Hepatitis	A	31
	C	1, 2, 5
Disseminated	A	31
	B	3, 7, 11, 21
	D	30, 43-47
None (???)	A	18
	D	9, 10, 13, 15 17, 20, 22-29, 33, 36, 38

Table 3

Serotype	Tail oligonucleotide	Knob oligonucleotide
4	A	1
8	B	2
9	B	2
12	E	3
16	C	4
19p	B	2
28	B	2
32	B	2
36	B	2
37	B	2
40-1	D	5
40-2	D	6
41-s	D	5
41-1	D	7
49	B	2
50	B	2
51	C	8

A: 5'- CCC GTG TAT CCA TAT GAT GCA GAC AAC GAC CGA CC- 3'(SEQ. ID. NO. __)

B: 5'- CCC GTC TAC CCA TAT GGC TAC GCG CGG- 3'(SEQ. ID. NO. __)

C: 5'- CCK GTS TAC CCA TAT GAA GAT GAA AGC- 3'(SEQ. ID. NO. __)

D: 5'- CCC GTC TAC CCA TAT GAC ACC TYC TCA ACT C- 3'(SEQ. ID. NO. __)

E: 5'- CCC GTT TAC CCA TAT GAC CCA TTT GAC ACA TCA GAC- 3'(SEQ. ID. NO. __)

Table 3 (cont.)

1: 5"- CCG **ATG CAT** TTA TTG TTG GGC TAT ATA GGA - 3'(SEQ. ID. NO. __)

2: 5'- CCG **ATG CAT** TYA TTC TTG GGC RAT ATA GGA - 3'(SEQ. ID. NO. __)

3: 5'- CCG **ATG CAT** TTA TTC TTG GGR AAT GTA WGA AAA GGA - 3'(SEQ. ID. NO. __)

4: 5'- CCG **ATG CAT** TCA GTC ATC TTC TCT GAT ATA - 3'(SEQ. ID. NO. __)

5: 5'- CCG **ATG CAT** TTA TTG TTC AGT TAT GTA GCA - 3'(SEQ. ID. NO. __)

6: 5'- GCC **ATG CAT** TTA TTG TTC TGT TAC ATA AGA - 3'(SEQ. ID. NO. __)

7: 5' - CCG **TTA ATT AA**G CCC TTA TTG TTC TGT TAC ATA AGA A - 3'(SEQ. ID. NO. __)

8: 5'- CCG **ATG CAT** TCA GTC ATC YTC TWT AAT ATA - 3' (SEQ. ID. NO. __)

Table 4

Adenovirus	Virus particles/ ml	Infectious units/ ml
Ad5Fib5	2.2×10^{12}	6.8×10^{11}
Ad5Fib12	4.4×10^{12}	1.9×10^{12}
Ad5Fib16	1.4×10^{12}	3.0×10^{10}
Ad5Fib17	9.3×10^{11}	9.5×10^{9}
Ad5Fib28	5.4×10^{10}	2.8×10^{8}
Ad5Fib32	2.0×10^{12}	1.1×10^{12}
Ad5Fib40-S	3.2×10^{10}	1.0×10^{10}
Ad5Fib40-L	2.0×10^{12}	6.4×10^{11}
Ad5Fib49	1.2×10^{12}	4.3×10^{11}
Ad5Fib51	5.1×10^{12}	1.0×10^{12}

Table 5

Celline	Ad5Fiber5	Ad5Fiber12	Ad5Fiber16	Ad5Fiber28	Ad5Fiber32	Ad5Fiber40-S	Ad5Fiber40-L	Ad5Fiber
A549	54186	2	283339	3556	46635	84562	407130	2
K562	1	5	109688	7915	30958	1086	1907	1524
SupT1	3926082	606032	14553005	855043	80834	ND	686546	77
GM09503	506	4	117094	1858	39652	52759	609	4
1^0 chorionvilli	75	147	1026757	203114	9756	ND	1688026	49
1^0 Amnionvilli	8420131	4975463	6991792	37512	3313879	ND	5250524	4081
HEPG2	108612409	11428921	19315715	962463	3844661	ND	90713451	23894
HeLa	6838148	510784	776984	13571	15600	1551397	1694919	103
CEM	93	6	1600	0	69	9	18	6.5
Synoviocytes	103	ND	9936417	ND	ND	ND	ND	ND
Smooth muscle cells	19019	664	816381	621	ND	ND	38632	ND

Table 6

					subgroup A	Subgroup B	Subgroup C	Subgroup D	Subgroup F
Celline	$a_v b3$	$a_v b5$	CAR	MHC class I	Ad5Fiber12	Ad5Fiber16	Ad5Fiber5	Ad5Fiber 32	Ad5Fiber40-L
A549	17%	98%	100%	ND	Low	High	High	High	High
K562	12%	55%	0%	15%	Low	High	Low	High	High
GM09503	20%	50%	0%	100%	Low	High	Low	High	Low
CEM	0%	0%	3%	100%	Low	High	Low	Low	Low
SupT1	5%	1%	70%	100%	High	High	High	High	High
Smooth muscle cells	100%	70%	0%	15%	Low	High	Low	ND	Low
HUVEC	100%	15%	10%	90%	ND	High	Low	ND	ND
Synoviocytes	30%	40%	0%	100%	ND	High	Low	ND	ND
1^0chorionvilli	100%	0%	12%	100%	Low	High	Low	Low	High
HepG2	0%	10%	100%	80%	High	High	High	High	High